UNIVERSIDADE FEDERAL DE SANTA CATARINA

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS GENÉTICOS VEGETAIS

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EMBRIOGÊNESE SOMÁTICA EM PUPUNHEIRA (*Bactris gasipaes*), BUTIÁ-DA-SERRA (*Butia eriospatha*) e AÇAÍ (*Euterpe oleracea*): ISOLAMENTO E CULTURA DE PROTOPLASTOS, AVALIAÇÕES BIOQUÍMICAS E OTIMIZAÇÃO DAS CONDIÇÕES DE CULTURA

Dissertação apresentada ao Programa de Pós-graduação em Recursos Genéticos Vegetais, da Universidade Federal de Santa Catarina, como parte do requisito para obtenção do título de Mestre em Ciências, área de concentração em Recursos Genéticos Vegetais.

Orientador: Prof. Dr.
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CARACTERIZAÇÃO DE EMBRIOGÊNESE SOMÁTICA EM PUPUNHEIRA (Bactris gasipaes), BUTIÁ-DA-SERRA (Butia eriospatha), E AÇAÍ (Euterpe oleracea), POR MEIO DE CULTURA DE PROTOPLASTOS, ANÁLISE BIOQUÍMICA E OTIMIZAÇÃO DE CULTURA / Joseph Francis Ree ; orientador, Miguel Pedro Guerra - Florianópolis, SC, 2015.
157 p.

Dissertação (mestrado) - Universidade Federal de Santa Catarina, Centro de Ciências Agrárias. Programa de Pós-Graduação em Recursos Genéticos Vegetais.

Inclui referências

1. Recursos Genéticos Vegetais. 2. Embriogênese Somática. 3. Arecaceae. 4. Micropropagação. 5. Protoplasto. I. Guerra, Miguel Pedro. II. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. III. Título.

EMBRIOGÊNESE SOMÁTICA EM PUPUNHEIRA (*Bactris gasipaes*), BUTIÁ-DA-SERRA (*Butia eriospatha*) e AÇAÍ (*Euterpe oleracea*): ISOLAMENTO E CULTURA DE PROTOPLASTOS, AVALIAÇÕES BIOQUÍMICAS E OTIMIZAÇÃO DAS CONDIÇÕES DE CULTURA

Esta Dissertação foi julgada adequada para obtenção do Título de "Mestre" em Recursos Genéticos Vegetais",e aprovada em sua forma final pelo Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina

Florianópolis, 6 de Fevereiro de 2015.

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Dedicated to the memory

of my grandfather, Joseph Ree

March 21, 1927-April 14, 2014

Acknowledgements

I thank, first and foremost, my parents, Paul and Kathy, my sister Meghanne, my grandma Ruth, my grandpa Frank, and all my family for their love and support from a hemisphere away.

I thank Joana Duarte Madalena, who has always been there to push me to achieve my goals and help me grow into a better person.

I thank the parents of Joana Duarte Madalena, Nelson Amâcio Madalena and Patrícia Duarte, who have been incredibly generous both with their support and friendship. I'd also like to thank her extended family, especially her grandparents Narbal José Duarte and Leni Angentil Duarte, who welcomed and accepted me from the very minute I first met them.

I thank my professor, Dr. Miguel Pedro Guerra for allowing me to work in such an interesting and exciting area of research. I'd like to thank him further for his keen insights, his patience, and explosive excitement at new data and information.

I thank Dr. Gabriela C. Cangahuala-Inocente for her help in teaching me many protocols essential for biochemical analysis.

I thank Hugo and Leila for their assistance in their help with several areas of this work and for helping me obtain the enzymes and plates used during the protoplast work.

I thank Andre for his assistance in several steps of the biochemical analysis.

I thank Samanta Siqueira de Campos for working with me to induce somatic embryogenesis in *Butia eriospatha* somatic embryos.

I thank Joana Duarte Madalena, Ramon Felipe Scherer, and Guilherme Telésforo Osório for help with writing technical Portuguese writing

I thank my friends Daniel, Daniela, Ramon, Francis, Liliana, Heindrich, Charlotte, Desi, Lilian, Marcia, Thiago, Montagna, and many others for their friendship.

I thank my many colleagues in the Laboratorio de Fisiologia do Desenvolvimento e Genética Vegetal for their comradery and mutual respect.

I thank Berna for helping me with all the paperwork I needed, starting with obtaining the documents required to obtain a student visa.

Special thanks to UFSC and the RGV for their top-notch professors. I'd like to thank UFSC and the RGV for allowing this work to be a possibility and for accepting me as a foreign student.

I'd like to thank CAPES for the financial support for me and my work during these last two years.

List of Abreviations

2-ip- 2-isopentenyladenine

ABA- Abscisic acid

AC- Activated charcoal

DW- Dry weight

FDA= Fluoroscein diacetate

FW- Fresh Weight

GA3- Gibberellic acid

HE- Highly embryogenic

IAA- Indole-3-acetic acid

IBA- Indole-3-butyric acid

LE- Low embryogenic

MW- Morel and Wetmore vitamins

MS- Murishige and Skoog media

NAA-1-naphthaleneacetic acid NAA

NE-Non-embryogenic

PEM-polyembryogenic mass

PGR- Plant Growth Regulator

ROS- Radical Oxidative Species

SE-Somatic embryogenesis

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General summary

Somatic embryogenesis (SE) is a powerful technology that is useful for commercial propagation, scientific study, germplasm conservation, and has potential applications in reforestation attempts in damaged ecosystems. Many organisms show similar traits during SE, such as the expression of commonly-found gene homologs, development of specialized tissues similar to zygotic embryogenesis, and response to certain growth regulators. To summarize these trends, a review article was written (Chapter 1 of this thesis). A literature analysis of SE in the palm family, Arecaceae, suggests similar trends across palm species. Both zygotic embryos or palm shoot meristems tended to respond well to culture media composed of Murashige and Skoog salts, vitamins, 3% sucrose, exogenous activated carbon, and high concentrations of auxins, such as 2,4-D or picloram. Embryo development was usually stimulated through subculture onto media with reduced auxins and addition of cytokinin, such as BAP and 2-ip and then embryos could be frequently converted on media without growth regulators. In addition to micropropagation, many studies focused on the biochemistry and gene expression of developing palm somatic embryos. Histological studies revealed common trends, such as similar morphology of meristematic vs. callus and epidermal cells. One of the technologies that several research groups had evaluated was protoplast culture. Protoplasts, cells with their cell walls removed, are of scientific interest due to both being able to regenerate into larger cultures and the ability to create hybrids through somatic fusion of two different protoplasts. This technology has only been investigated in several large-scale economic species, such as oil palm (*Elaeis guineensis*) or date palm (*Phoenix dactyilifera*), and increased understanding of trends might lead to further development of protoplast culture as a whole (Chapter 2). To understand protoplast isolation and culture, previously-established peach palm (*Bactris gasipaes*), butiá-da-serra (*Butia eriospatha*), and açaí (*Euterpe oleracea*) cultures were treated with an enzyme solution composed of 2% w/v cellulase, 0.5% hemicellulase, and 0.5% pectinase and then either incubated stationary for 6, 12, 18, or 24 hours or on an orbital shaker at 45 rpm for 3, 6, 12, or 24

hours in the dark at 25±2 °C. Stationary incubation did not provide large amounts of protoplasts in comparison to incubation done on an orbital shaker. The greatest numbers of protoplasts per species were $5.50\pm0.68x10^5$ and $1.22\pm0.13x10^6$ protoplasts/ gram FW for peach palm and açaí after six hours of orbital shaking and $5.36\pm2.23x10^5$ cells/gram FW for butiá after 24 hours of orbital shaking. Both peach palm and açaí saw dramatic decreases in protoplast yield after later incubations while the amount of visible cellular debris increased, possibly showing a potential reason for the decreased cell yield as cellular debris might lyse cells in motion. Protoplast viability remained high (>70%), except for açaí protoplasts, which decreased rapidly during orbital incubation. Protoplasts were cultured either using the agarose bead or the alginate bead method. Protoplasts cultured in alginate beads were subjected to six types of liquid media containing 0, 1, or 10µM picloram either with or without 2µM 2-ip. Auxin was not shown to be essential for causing cell division in several occasions, however cell division was more frequent and occurred earlier in media with increasing levels of picloram. 2-ip was not found to have a major impact. Microcolonies were observed to form in alginate beads with 10µM picloram and 2µM 2-ip, however visible colonies were only formed twice in agarose beads. Further optimization would be required to achieve regeneration of whole plants, however there are many trends in other species, such as use of nurse cultures, higher cell density during culture, and type of culture method, which can be pursued. The biochemistry of peach palm cultures with different capacities for SE, high embryogenic (HE), low embryogenic (LE), and non-embryogenic (NE), were investigated for dry weight, protein, sugar, and starch content (Chapter 3). It was found that both HE and NE cultures had similar dry weights, but water made up a larger proportion of LE cultures. Because of this difference, protein, sugar, and starch amounts were evaluated in terms of both fresh weight and dry weight. HE contained slightly higher amounts of protein than NE cultures, but both tissues contained higher proteins contents than LE cultures, even after adjusting for dry weight. Starch levels were comparable between HE and NE cultures, but LE cultures contained significantly fewer starch reserves. Sugar levels were too low to have their amounts calculated using a glucose standard curve, however their absorbance readings showed HE and NE cultures had the

about the same values, while once again LE cultures contained fewer reserves. These data suggest that the different growth behavior of the different tissues may not reflect overall protein amounts, but rather the types of proteins being expressed. Additionally, the large amounts of starch growth, along with the distinct morphology of NE tissues might suggest a type of organogenesis. Additionally, it is possible that the lack of energy reserves found in LE tissue is related to its rapid growth. Organized tissue formations were also observed in LE tissue, possibly suggesting that *in vitro* organogenesis extends beyond simply somatic embryos, roots, and shoots. Additionally, SE optimization was investigated in peach palm, butiá, and açaí cultures. Peach palm tissue lines 'G3' were placed on media containing either MS or modified MS salts and media containing 1mM spermidine, spermine, or no polyamines. No difference was detected in the number of somatic embryos recovered from MS or modified MS cultures, however either polyamine had a negative effect. Peach palm 'G2' cultures were placed on media containing either 3% sucrose or an equal mix of sucrose, glucose, or sorbitol with no difference in total numbers of recovered embryos. Fast-growing friable butiá cultures were subjected to numerous types of media, including both solid and liquid media, media containing different concentrations of auxins, cytokinins, ABA, GA3, sucrose, and activated charcoal in both light and dark, but no somatic embryos were induced. Additionally, partial dehydration did not induce improved peach palm multiplication nor butiá SE. However, 40% and 27.5% placed on MS media containing 2.5g/L activated charcoal and 300µM or 450µM, respectively, picloram responded with tissue growth similar to that found in peach palm highly embryogenic tissue. Açaí multiplication was greatly improved through optimizing multiplication media with addition 2.5g/L activated charcoal and increased picloram up to 50µM from 10µM. The effects of the activated charcoal led to 400% reduction in number of oxidized explants, an increase in number of explants producing embryos, embryos per explant, number of embryos producing polyembryogenic masses, and number of polyembryogenic masses per explant. However, instances of organogenic root growth and increased callogenesis were observed on açaí explants placed on media with activated charcoal, which suggests that the decreased effect of auxin caused by activated charcoal

can reduce embryogenic potential. Together, these works contribute to better understanding the trends found in multiple palms, factors involved in the valuable technology of protoplast culture, the biochemical characteristics of several types of peach palm tissue with different embryogenic potential, and cultural optimization, especially for the economically valuable açaí palm.

Keywords: Arecaceae, Somatic Embryogenesis, Protoplast, Recalcitrance, Micropropagation

Resumo

A embriogênese somática (ES) é um métedo eficiente à propagação massiva de plantas e à conservação de germoplasma. Em nível básico ela se configura como um modelo biológico para o aprofundamento de estudos de morfogênese, fisiologia, bioquímica e genética de plantas. Muitos organismos apresentam características similares durante a ES, tais como a expressão de genes homólogos, o desenvolvimento de tecidos especializados semelhantes ao observado na embriogênese zigótica e as respostas a certos reguladores de crescimento. Para abordar profundamente estas características associadas à ES de palmeiras neotropicais foi elaborada uma ampla revisão sobre este tema no primeiro capítulo desta dissertação. De uma forma geral esta revisão mostrou que embriões zigóticos e/ou meristemas apicais têm sido os explantes mais empregados, mostrando-se responsivos ao meio de cultura baseado na formulação salina de Murashige e Skoog suplementado com vitaminas de Morel, 3% de sacarose, carvão ativado e concentrações elevadas de auxinas, principalmente 2,4-D ou picloram. Em seguida, o desenvolvimento embrionário normalmente é estimulado em meios de cultura suplementados com teores reduzidos destas auxinas e com o emprego de citocininas, notadamente BAP e 2-ip. Por fim, os embriões são convertidos em plântulas em meios isentos de reguladores de crescimento. Além da micropropagação, muitos estudos concentraram-se na expressão de genes e na bioquímica do desenvolvimento de embriões somáticos de palmeiras. Estudos histológicos sugeriram tendências comuns em diferentes espécies durante a morfogênese *in vitro*, tais como morfologia semelhante dos ápices caulinares meristemáticos, calos e células epidérmicas. No segundo capítulo estudou-se o emprego de culturas de protoplastos em três espécies de palmeiras, pupunha (*Bactris gasipaes*), butiá-da-serra (*Butia eriospatha*) e açaí (*Euterpe oleracea*). Protoplastos são células com suas paredes celulares removidas e apresentam interesse tanto pela sua capacidade regenerativa quanto pela possibilidade da geração de híbridos através da fusão somática de dois protoplastos diferentes. Esta tecnologia tem sido investigada em várias espécies de palmeiras, tais como dendê (*Elaeis guineensis*) e tamareira (*Phoenix dactyilifera*). Na presente dissertação, culturas embriogênicas previamente estabelecidas de pupunha, butiá-da-serra e açaí foram tratadas com uma solução enzimática composta por 2% de celulase, 0,5 % de hemicelulase e 0,5 % de pectinase e em seguida elas foram

incubadas no escuro a 25 ± 2 ° C durante 6, 12, 18, ou 24 horas sem agitação ou num agitador orbital a 45 rpm para o isolamento de protoplastos. As maiores quantidades de protoplastos foram obtidas através da incubação em agitador orbital, sendo $5.50\pm0.68x10^5$ e $1.22\pm0.13x10^6$ protoplastos / grama de peso fresco de pupunha e açaí, respectivamente, depois de seis horas de incubação, e $5,36 \pm 2.23x10^5$ células / grama de peso fresco para butiá após 24 horas incubação. Culturas de pupunha e açaí mostraram diminuição de rendimento de protoplastos após seis horas de incubação, enquanto a quantidade de resíduos celulares visíveis aumentou. A viabilidade de protoplastos manteve-se elevada (> 70%), com exceção de protoplastos de açaí, cuja viabilidade diminuiu rapidamente após seis horas na incubação orbital. Os protoplastos foram cultivados usando o método de gotas de agarose ou de alginato, sendo submetidos a seis tipos de meios líquidos contendo 0, 1, ou 10 μM de picloram com ou sem 2μM de 2-ip. Picloram não se mostrou essencial para as divisões celulares, as quais, no entanto foram mais frequentes e ocorreram mais cedo em resposta a meios com níveis crescentes do mesmo. Foram observadas microcolônias se formando nas gotas de alginato com 10 de μM picloram e 2 μM de 2-ip, no entanto colonias visíveis foram formados apenas em duas esferas de agarose. No terceiro capítulo desta dissertação o incremento de massa seca e os teores de proteínas, açúcares e amido foram investigados em culturas de pupunha com diferentes capacidades para ES: alta capacidade embriogênica (ACE), baixa capacidade embriogênica (BCE), e não-embriogênica (NE). Culturas de ACE e NE apresentaram incrementos de massa seca semelhantes e que foram maiores queo incremento de massa seca em culturas de BCE. Culturas de ACE apresentaram teores ligeiramente mais elevados de proteínas do que as culturas NE, contudo ambas continham maiores teores de proteínas do que as culturas de BCE. Níveis de amido foram semelhantes entre culturas de ACE e NE e que foram maiores que os níveis de amido em culturas de BCE. Níveis de açúcares foram demasiadamente baixos para terem seus valores calculados utilizando uma curva padrão de glicose, porém as leituras de absorbância mostraram que as culturas de ACE e NE apresentaram valores médios semelhantes e superiores aos níveis encontrados em culturas de BCE. Estes dados sugerem que os diferentes comportamentos de crescimento das diferentes culturas podem não refletir as quantidades totais de proteínas, e sim os tipos de proteínas a serem expressas. A grande quantidade de amido nas culturas NE, juntamente com a morfologia

distinta destas culturas, pode sugerir uma rota regenerativa baseada na organogênese. Além disso, é possível que a falta de reservas de energia nas culturas de BCE esteja relacionada com o seu rápido crescimento. Formações de tecidos organizados também foram observadas nas cultura de BCE, isto pode sugerir que a morfogênese *in vitro* não inclui apenas os embriões somáticos, calos, raiz e plântulas, mas pode incluir outros tipos de tecidos. No quarto capítulo buscou-se a optimização da ES em culturas de pupunha, butiá e açaí. Culturas de pupunha 'G3' foram cultivadas em meios de cultura compostos pelos sais de MS ou MS modificado, suplementados com 1 mM de espermidina, 1 mM de espermina, ou sem poliaminas. Não houve diferença no número de embriões somáticos regenerados a partir dos meios de MS ou MS modificado, no entanto, ambas as poliaminas apresentaram efeitos negativos para a formação de embriões. Culturas de pupunha 'G2' foram inoculadas em meio de cultura contendo 3% de sacarose ou uma mistura igual de sacarose, glicose, frutose, e sorbitol, não revelando diferenças em termos de números totais de embriões regenerados. Embriões zigóticos de butiá inoculados em meio MS contendo 3% sacarose, 2,5 g / L de carvão ativado e 300 μM ou 450 μM de picloram responderam com a formação de calos com aparência embriogênica similares aos observados em pupunha, 40% e 27,5% respectivamente. No entanto, culturas friáveis de butiá foram submetidas a vários tipos de meios de cultura, incluindo meios sólidos e líquidos; diferentes concentrações de auxinas, citocininas, ABA, GA3, sacarose e carvão ativado,e ausência ou presença de luz durante o cultivo, porém, em nenhum dos ambientes proporcionados ocorreu a formação de embriões somáticos. Além disso, desidratação parcial não induziu melhorias na multiplicação das culturasde butiá, o mesmo foi observado em culturas de pupunha. A multiplicação das culturas de açaí foi melhorada com a adição 2,5 g / L de carvão ativado e aumento nos teores de picloram de 10 μM até 50 μM. O carvão ativado levou à redução de 400% no número de explantes oxidados, um aumento no número de explantes produzindo embriões, número deembriões formados por explante, e numero de massas poliembriogênicas por explante. No entanto, os casos de crescimento de raizes organogênicas e de calogênese observados a partir de explantes de açaí colocadas em meios com carvão ativado sugerem que o efeito da auxina diminui na presença de carvão ativado. Tomados em conjunto, os resultados da presente dissertação contribuem para uma melhor compreensão dos estudos da morfogênese *in vitro* de palmeiras, notadamente aqueles

associados aos fatores envolvidos na tecnologia de protoplastos, às características bioquímicas de culturas de pupunha com diferentes potenciais embriogênicos e à otimização dos protocolos regenerativos estudados, especialmente o de açaí.

Keywords: Arecaceae, Embriogênese Somática, Protoplasto, Recalcitrancia, Micropropagação

General objectives

The goal of this work was to elaborate on several aspects of somatic embryogenesis and its applications in three native Brazilian palm species. Using literature review, trends in somatic embryogenesis and experimental new applications were selected: protoplast isolation and culture, biochemistry of tissues with different embryogenic capacities, replications of successes in other studies, and synthesis of new ideas for media optimization.

Specific objectives:

Chapter 1: Collect and organize information about somatic embryogenesis as it applies to palms using all known published articles involving somatic embryogenesis in any way.

Chapter 2: Characterize factors relating to protoplast isolation and culture in three palm species, as well as to understand the requirements for early protoplast culture and regeneration.

Chapter 3: Collect biochemical data about high, low, and non-embryogenic peach palm tissues to look for a distinctive marker of embryogenic competence

Chapter 4: Replicate of past successes in media optimization in other species, as well as induce somatic embryogenesis in recalcitrant butia cultures, and solve the problem of widespread açaí tissue oxidation through optimization with activated charcoal

1. PALM (ARECACEAE) SOMATIC EMBRYOGENESIS

A review article submitted to the journal *In vitro* Cellular and Developmental Biology- Plant

Manuscript draft

Current status: Pier reviewed, undergoing revisions

Abstract
Palm tree somatic embryogenesis has become increasingly important for agriculture, especially for mass propagation of commercially-important species, such as oil palm, coconut, and date palm. Through careful research and painstaking optimization studies, many palm species, including elite cultivars, can now be regenerated *in vitro*, which, in comparison to other propagation methods, is the most suitable means of producing palms for human need. Elite genotypes, genetic gains from breeding programs, and species threatened with extinction can be preserved both through *in vitro* propagation, as well as through cryopreservation of somatic embryos. In addition, somatic embryogenesis has given researchers a tool to study the mechanics of embryogenesis, thereby allowing a greater understanding on plant physiology, biochemistry, gene expression, and epigenetics as a whole. However, somatic embryogenesis is a complex morphological pathway that is reliant on many factors, such as tissue culture environment, genotype-dependent factors, epigenetic state, type and concentration of growth regulators, explant and health of explant tissue, and stress level. In this review, we summarize the advancements made in understanding palm somatic embryogenesis and its applications.
Keywords
Somatic embryogenesis, Arecaceae, palms, *in vitro* regeneration, micropropagation

Introduction

Palms in agriculture

Few plant families have given humans a more varied assortment of natural products than the Arecaceae, the palm family. For example, coconuts (*Cocos nucifera*) are widely used in confections, cooking ingredients, and beverages, the starchy peach palm (*Bactris gasipaes)* fruit has been a staple diet of inhabitants of the Amazon Basin for centuries (Mora-Urpi et al., 1997), trunks and fibers from the rattan palms (*Calamus* spp.) can be woven into furniture and handicrafts (Hemanthakumar et al., 2013), oil from oil palm (*Elaeis guineensis)* fruits is one of the most used cooking oils in the world (Thuzar et al., 2011), and areca palm (*Areca catechu)* fruits are often chewed to release stimulants in several regions of Southeast Asia (Wang et al., 2006). Palm wood, fibers, and leaves have been traditionally used as

construction material for homes, furniture, baskets, ropes, and roofing (For a list of palms, their uses, and status of somatic embryogenesis (SE) protocols, see Table 1, for a more complete review of useful palms, see Haynes and McLaughlin, (2010). While the most widely cultivated crops, maize, rice, wheat, potato, and cassava, form the backbone of modern agriculture, it is undeniable that palms form an essential role in many industries. Indeed, with increasing global trade and population comes demand for more and varied products, including novel foods, efficient crops, pharmaceuticals, and luxury items. As such, agricultural technology must rise to meet economical pressure. Central to this technology is *in vitro* regeneration and, specifically to this review, somatic embryogenesis, the process of creating whole viable embryos from somatic tissues (Verdeil et al., 1994). This biotechnology, first described in palms by Rabechault et al., (1970) in oil palm cultures, allows the capture of selected genotypes, including elite cultivars, for commercial, scientific, and conservation purposes. Over the last several decades, hundreds of researchers from around the globe have unveiled new insights into palm embryogenesis and have developed SE induction protocols in 19 or more palm species encompassing dozens of cultivars and a vast unknown number of genotypes.

Table 1-1: A list of economically, culturally, scientifically, and environmentally interesting palms, their uses, conservation status, and tissue culture state

Species	Common name(s)	Native region	Current and traditional uses, notes	References	IUCN status	Published successful SE?
Acrocomia aculeata	Macaw palm, Grugru palm, Macaúba palm	Tropical regions of the Americas	• high oil yield, possible use as a biodiesel-producing crop • Sap fermented into an alcoholic beverage in southern regions of Mexico for thousands of years	Moura et al. (2010) Alcantara-Hernandez et al. (2010)	Unassessed	Yes
Areca catechu	Areca palm, Areca nut palm, Betel palm	Southeast Asia, Pacific islands, and East Africa	• In some Southeast Asian countries, the seed is chewed to release stimulants • ornamental	Wang et al. (2006) Staples and Bevacqua (2006)	Unassessed	Yes
Bactris gasipaes	Peach palm, Pupunha, Pejibaye, Chontaduro, Pijuayo	Amazon rainforest to Central America	• Starchy or oily fruits eaten, cooked, made into a fermented beverage, ground into a gluten-free flour, • heart of palm, considered a sustainable source due to multiple growing stems • Trunks used in construction of homes and crafts such as furniture, flooring, bows, arrows, fishing poles, and carvings • Leaves woven into roofing, baskets, clothing, • Roots have vermicide properties	Mora-Urpi et al., (1997)	Unassessed	Yes
Borassus flabellifer	Asian palmyra palm, Toddy palm, Sugar palm	Southeast Asia and India	• Sap used to make an alcoholic beverage • Stems and leaves to construct houses, • Sweet fruits widely consumed, possible pharmacological use from spirostane-type steroid saponins with antidiabetogenic activity	Yoshikawa et al (2007) Davis and Johnson (1987)	Unassessed	Embryogenic callus, no SE
Butia eriospatha	Butiá, Butiá-da-serra, Butiázeiro	South Brazil	• Ornamental • Fruits consumed and processed into flavorings for ice cream and jellies, seeds are edible with flavor like coconut	Claumann (2009), Noblick (1998)	Vulnerable	Embryogenic callus, no SE
Calamus spp.	Rattan palms (general term for *Calamus)*	India, Southeast Asia, Pacific Islands, Indonesia	• Wood and fiber used for furniture and handicrafts • Traditional use in Ayurvedic system of medicine • Oils extracted from the roots used as a perfume and for flavoring liquors	Goh et al. (2001) Hemantha kumar et al. (2012)	Least concerned to critically endangered	Yes

Caryota urens	kitul palm, fish tail palm, toddy palm, jaggery palm	India and Southeast Asia	• Sap from inflorescence yields a crude brown sugar, called jaggery, which is converted to toddy, an alcoholic beverage	Ranasinghe et al. (2012) Zoysa (1992)	unassessed	None
Ceroxylon quindiuense	Palma de Cera del Quindio, Quindio wax palm	Colombia	• Wax harvested from trees for candles • Trunks had been locally used for construction, power line poles, and water pipes. • National tree of Colombia	Madriñán (1995) Bernal (1998)	Vulnerable	None
Cocos nucifera	Coconut, Coconut palm	Worldwide tropics	• Coconut water, the liquid endosperm, is a beverage • Coconut milk made from grated coconut flesh is a culinary ingredient, especially in southeast Asia • Oil can be used for cooking • Sap can be made into jaggery, toddy, syrup, and alcohol • Heart-of-palm is cultivated and sprouts are also consumed • The fiber from the husk of a coconut, the coir, is used in making rope, mats, sacks, caulking for boats, and stuffing for mattresses • Husks and shells can be made into cups and bowls, as well as used to make charcoal and activated carbon • Coconut has also been used in traditional medicine in multiple cultures, with some modern pharmacological research into usable new drugs • Coconut trunks can be used for construction and handicrafts.	Chan and Elevitch (2006) Adkins et al. (2006)	unassessed	Yes
Cyrostachys renda	Sealing wax palm, Red sealing wax palm, Lipstick palm	Malay Peninsula, Sumatra, and Borneo	• Highly prized ornamental due to brilliant red leaf sheaths	Heatubun et al. (2009)	Unassessed	None
Elaeis guineensis	Oil palm, African oil palm	West and southwest Africa	• Produces palm oil for cooking, in competition with soybean for most vegetable oil production • Sap can be made into a fermented beverage	Lasekan and Otto (2009)	unassessed	Yes, multiple cultivars
Euterpe edulis	Juçara, Palmiteiro	Southeast Brazil	• Heart of palm production • Pulp of the fruits can be eaten fresh or added to a beverage	Fantini and Guries (2007)	Unassessed	Yes

Euterpe oleracea	Açaí, Açaí palm	Amazon rainforest	• The sweet pulp of the fruit is a popular flavoring agent. Pulp may also be consumed directly after the seed is removed. • Heart of palm	Muniz-Miret et al. (1996)	Unassessed	Yes
Hyophorbe amaricaulis	Loneliest palm	Mauritius	• Single remaining individual of the species located in the Curepipe Botanic Garden in Curepipe, Mauritius	Bachraz and Strahm (2000)	Critically endangered	None
Hyophorbe lagenicaulis	Bottle palm	Mauritius	• Critically-endangered species with few remaining wild trees. Use as ornamental plant	Sarasan et al. (2002) Page (1998)	Critically-endangered	SE induced, embryos failed to convert
Hyphaene thebaica	Doum palm, Gingerbread tree, Doom palm	Upper Egypt, Sudan, Kenya, Tanzania	• Fiber and leaflets traditionally used for weaving into baskets, mats, brooms • Trunks used in construction of homes • Fruit is edible, evidence of its cultivation during ancient Egypt and found in ancient tombs • Fruits contain compounds with hypocholesterolemic effects	Janick (2000) Aremu and Fadele (2011) Hetta and Yassin (2006)	Unassessed	None
Jubaea chilensis	Chilean wine palm	Chile	• Sap used in for traditional palm "honey" and fermented beverages • Seeds, called coquito nuts, are edible, having similar taste to coconut	Haynes and McLaughlin (2000) González (1998)	Vulnerable	None
Lodoicea maldivica	Sea coconut, Coco de mer	Praslin and Curieuse in the Seychelles	• The seed, the largest seed in the plant kingdom, is often sold in the tourist trade due to its unique shape	Rist et al. (2010) Fleischer-Dogley et al. (2011)	Endangered	None
Mauritia flexuosa	Moriche palm, ita, buriti, canagucho, aguaje	Brazil, Peru, Venezela, Colombia, Ecuador, Bolivia	• Oil rich in monounsaturated fatty acids, use in traditional medicines • Fruit is consumed or added to ice cream and cold drinks, rich in vitamin A and E	França et al. (1999) Manzi and Coomes (2009)	Unassessed	None

					Silva et al. (2009)		
Metroxylon sagu	True sago palm	New Guinea, Indonesia, Malaysia	•	Industrial starch production from its trunk, where the plant accumulates vast amounts to be used for fruit production	Alang and Krishnapillay (1987) Kjaer et al. (2004)	Unassessed	Yes
Nypa fruticans	Nipa palm	South East Asia, India, Pacific Islands	•	Leaves used in roofing and walls	Hamilton and Murphy (1988) Ellison et al. (2010)	Least concern	None
			•	Fibers can be made into rope,			
			•	Leaflets can be woven into hats, umbrellas, raincoats, baskets, mats, bags			
			•	Young seed endosperms used to flavor ice cream in Malaysia			
			•	Hardened endosperm used as vegetable ivory and buttons			
			•	Various parts of the plant used in traditional medicine			
			•	Sap used to make alcohol, vinegar, and fermented beverages			
Phoenix dactylifera	Date, Date palm	Egypt to the Middle East	•	Fruits can be eaten fresh, dried, processed into syrup, vinegar, and alcohol	Chao and Kruger (2007) Nixon (1951)	unassessed	Yes, multiple cultivars
			•	Trunks can be used for timber, fuel, and wood			
			•	Fibers can be made into bags, baskets, camel saddles, cords, fans, food covers, furniture, mats, ropes, and twine			
			•	Leaves may be used to make roofing			
			•	Leaf ribs can be used to build boats and fishing traps			
			•	Heart-of-palm			
			•	Use of dates in traditional medicine			
			•	Ecological use in preventing desertification.			
Phytelephas aequatoriali s	Ecuadorean ivory palm	Ecuador	•	The seeds, called tagua seeds, are a source of vegetable ivory used for buttons and carvings	Runk (1997) Montúfar and Pitman (2003)	Near threatened	None
			•	Heart of palm			
			•	Leaves used for roofing			
			•	Trunks used as fuel			
			•	Tagua powder marketed as industrial abrasive for "green" products			
Pritchardia remota	Nihoa pritchardia, Nihoa fan palm, Luo'lu	Nihoa island of Hawai'i	•	Ornamental	Perez et al.(2008) Gemmill (1998)	Endangered	None

Raphia spp.	Raffia palm	Tropical regions of Africa and Madagascar, one species, *R. taedigera,* occurs in South America	• Oils extracted from the kernel for cooking and biodiesel • Long leaves used for baskets and tie materials • Palm wine can be made from raphia palm • *R. hookeri* gum potentially inhibits corrosion of steel in sulfuric acid in a range of temperatures	Umoren et al (2009)	Many unassessed, few vulnerable	None
Rhopalostylis sapida	Nikau	New Zealand	• Young shoots and heart-of-palm eaten • Ornamental • Leaves can be woven into baskets and as roofing	Brooker et al. (1989) Dowe (1998)	Least concern	No
Sabal palmetto	Cabbage palm, Palmetto, Cabbage palmetto, Sabal palm	Gulf Coast of the USA, Cuba, The Bahamas	• Heart of palm • Seeds were used by North Americans natives for soothing headaches and lowering fevers • Wood and fibers used in home construction and crafts, such as baskets, nets, arrows, and mats	Bennet and Hicklin (1998) Gallo-Meagher and Green (2002)	Unassessed	Yes
Salacca glabrescens	Jungle salak, Salak hutan, Sala	Thailand and Peninsular Malaysia	• The pulp of the fruit is edible	Zulkepli et al., (2011) Lim (2012)	unassessed	Embryogenic callus, no SE
Trachycarpus fortunei	Windmill palm, Chusan palm	South-Central China	• Fibers used to make rope, thatch, mats, mattresses, and traditional raincoats • Trunks used as house pillars • Seeds used as animal fodder, high quality wax can be extracted from seeds • Leaves woven into fans, hats, chairs, and roofing • Used in some Chinese traditional medicine	Essig and Dong (1987) Zhai et al (2011)	Unassessed	None

Palm somatic embryogenesis

Uses of palm SE

Mass propagation and capture of genetic gains from breeding programs is the foremost application of SE for commercial sectors. In a similar way, SE offers a means for preserving population genetic diversity as part of an *ex situ* conservation program. A foremost example of tissue culture success in conservation was demonstrated by Sarasan et al., (2002), who induced SE in the nearly-extinct bottle palm (*Hyphorbe leganicaulis*), which, according to 1996 report of the International Union for Conservation of Nature and Natural Resources (IUCN), had fewer than ten wild plants left in the world. The authors demonstrated successful *in vitro* germination of zygotic embryos, improved plantlet acclimation with paclobutrazol, and formed somatic embryos. However, none of the somatic embryos were reported to give rise to entire regenerated plants. While not a perfect outcome, it is essential to understand that SE is a complex process relying on many factors, such as genotype, explant source, and tissue culture conditions.

Somatic embryogenesis induction

One of the foremost factors to successful SE induction is stress. Stress, either due to hormone types and concentrations, osmotic pressure, tissue culture conditions, gene expression, oxidative damage, or the effects of ethylene, has been characterized as an essential factor in SE induction (for review, see Karami and Saidi, (2010). Osmotic pressure is a major source of stress involved in dedifferentiation, which is heavily influenced by the concentration of carbohydrates added to culture media (for review, see Yaseen et al., 2013). Osmotic stress may alter the plasmodesmatal connections between cells (Schulz, 1995), thus disrupting communication regulates cell fate, but disruption of signaling by cell isolation can cause re-differentiation (McLean et al., 1997). SE is induced either directly (without an intermediate callus phase) or indirectly (with an intermediate callus phase). For example, Jayanthi et al. (2011) achieved direct SE on the surface of oil palm zygotic embryos. In another study, coconut zygotic embryo plumules grew embryogenic calli, which later developed immature globular embryos (Chan et al., 1998). Both routes led to tissue growth through morphological steps characteristic of zygotic embryogenesis (ZE). However, unlike ZE, which begins with a single fertilized ovule cell, SE has been described as having singular or

multicellular origins depending on the study. Both Saénz et al., (2006) and Chan et al., (1998) reported multicellular SE origins in coconut cultures. Verdeil et al., (1994), reported single-cell origins in coconut cultures, but, in previous work, Verdeil et al. (1989), reported multicellular origins. Kanchanapoom and Domyoas (1999) observed single-cell origin of oil palm embryoids in the subepidermis along the surface of callus clumps. Oil palm proembryos were seen to have multicellular origin (Balzon et al., 2013). In two different coconut cultivars, one was observed to have multicellular SE origins, while the other had unicellular origins (Dussert et al., 1995). Therefore, both single and multicellular somatic embryo origins may occur depending on genotype and culture conditions. There is little to suggest that either singular or multicellular origins are beneficial or detrimental over the other, however, Schwendiman et al., (1990) reported that oil palm somatic embryos developing from a unicellular origin always aborted. SE is described as being an asynchronous process in many palms, such as juçara (*Euterpe edulis)* (Guerra and Handro, 1998), and açaí (*Euterpe oleracea*) *(*Scherwinski-Pereira et al., 2012a*),* allowing the continuous development of new embryos over time.

Genotypes and regeneration

Genotype often dictates regeneration success *in vitro*. Some genotypes regenerate readily, some inefficiently, and others can be considered completely recalcitrant; some regenerate only callus and others fail to grow any tissue. Recalcitrance to *in vitro* regeneration overshadows many studies and is most certainly not confined to Arecaceae, as shown in plant such as *Quercus robur* (Toribio et al., 2004), *Feijoa sellowiana* (Guerra et al., 2001), and *Picea abies* (Arnold et al., 1996). However, similarities to tissue culture response are not impossible, as shown in a study of SE in 'Jihel' and 'Bousthami noir' date palm (*Phoenix dactylifera*) cultivars (Zouine et al., 2004). Zouine et al., (2004) found that, despite some variation, similar trends were presented among a set of eight different liquid media treatments, wherein the two most effective treatments were the same for both cultivars. Many more studies show far reaching differences between genotypes. In a comparison between six date palm cultivars, the 'Muzati' and 'Khalasah' cultivars far surpassed the 'Barhee' cultivar for embryogenic callus formation, SE induction, maturation, and conversion (Aslam et al., 2011). Genotype-specific responses to tissue culture components, such as type of basal salt mixture type in date palm

(Al-Khayri, 2011b), growth regulator type and concentration in varieties of areca palm (Karun et al., 2004) and four cultivars of date palm (Sané et al., 2006), and response to exogenous silver nitrate among five date palm genotypes (Al-Khayri and Al-Bahrany, 2004) have been reported. Among two varieties of interspecific *E. oleifera x E. guineensis* hybrids, Alves et al., (2011) reported that the percent of embryogenic callus formation on media supplemented with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) had an inverse relation between the two varieties, with one variety having its highest rate of calli formation at the lowest concentrations and decreasing as 2,4-D concentration increased. The opposite occurred in the other variety with increasing callus formation with increasing 2,4-D. These complex, often conflicting, results highlight the formidable research bottleneck that prevents use of many plants in tissue culture. However, there have been successful attempts in reducing recalcitrance in several plants, such as in *Gossypium hirsutum* (Wu et al., 2004), *Glycine max* (Bailey et al,. 1993), and *Hordeum vulgare* (Cho et al., 1998).

SE associated genes

Expression of certain genes has been tied to embryogenic competence. One such gene, Somatic Embryogenesis Responsive Kinase (SERK), is a highly conserved gene involved in SE across many types of plant species, such as the dicot angiosperm *Medicago trancatula* (Nolan et al., 2003) and the gymnosperm *Araucaria angustifolia* (Steiner et al., 2012). A SERK homolog was found to be expressed preferentially in embryogenic cells, but not in non-embryogenic cells, in coconut (Pérez-Núñez et al., 2009). Likewise, SERK cDNA transcripts were found in both *Elaeis guineensis x E. oleifeira* hybrid callus lines and zygotic embryos (Angelo et al., 2013). Three SERK genes, designated, PhSERK1, PhSERK2, and PhSERK3 were identified in embryogenic date cultures (Rekik et al., 2013). SERK homologs are therefore a possible common marker of embryogenic competence in palms. In coconut cultures, a homolog of a cyclin-dependent kinase (CDKA) gene, called CnCDKA, was found to be increasingly expressed within meristematic centers of embryogenic calli, but expression decreased during SE until the gene remained undetectable during embryo conversion (Montero-Cortés et al., 2010). Jouannic et al. (2007) reported the expression of an oil palm homolog of KNOX genes, EgKNOX1, in both somatic embryo and embryogenic calli tissue, while

no gene transcripts were detected in non-embryogenic tissue. Genes activated in response to SE include the Aux/IAA gene EgIAA9 isolated from oil palm (Ooi et al., 2012), whose expression decreased in response to increasing concentration of exogenous 2,4-D. These authors suggested that endogenous auxin levels may be greater in highly-embryogenic palms. Lin et al. (2009) constructed suppression subtractive hybridization (SSH) libraries with GO terms to find differentially-expressed genes during several stages of oil palm suspension cultures and found trends corresponding to other organisms, such as the expression of glutathione S-transferase, an enzyme involved in detoxifying oxidative-stress metabolites.

Epigenetic changes

Accompanying the activation of these genes are epigenetic shifts, such as chromatin remodeling and DNA methylation/demethylation, in response to both stimuli from tissue culture conditions and cellular dedifferentiation (for review, see Smulders and De Klerk, 2011). During *in vitro* regeneration, methylation levels rise and fall, as demonstrated in the angiosperm *Populus trichocarpa.* As explants underwent callogenesis, global methylation increased, but regenerated plantlets showed nearly equal pre-callogenesis methylation (Vining et al., 2013). According to Rival et al. (2013) oil palm cultures maintained over long periods of time showed increased methylation, potentially leading to loss in embryogenic potential. Wang et al. (2010) reported that long-term areca palm embryogenic cultures developed aberrant structures with increasing frequency over several years, however, it is not known if methylation was a factor. Methylation is one of several hypotheses of the reasons behind 'mantled' oil palm phenotypes (Jaligot et al., 2000). 'Mantled' phenotypes refer to abnormal oil palm floral organ development, resulting in partial or entire sterility of the plant, thus leading to overall yield loss (Corley et al., 1986). This phenotype is thought to arise through somaclonal variation arising from cellular reprogramming during the transition of somatic cells to embryogenic cells (Lucia et al., 2011). SHH was successfully employed in transcriptome analysis of markers distinguishing 'mantled' phenotypes from normal phenotypes by identifying two genes, EgFB1 and EgRING1, which encode proteolytic signaling complexes down regulated in 'mantled' phenotypes (Beulé et al., 2010). The potential problems caused by

somaclonal variation in oil palm, however, show the importance of further research into the cellular and genetic states of tissue during SE.

Initial cell division

Cells from multiple types of tissue have been observed to be the first to begin dividing. The first signs of cell division were from cells bordering vascular tissue on young date palm leaves (Sané et al., 2006). Embryogenic calli grew from outlying parenchymal cells of peach palm zygotic embryos while showing signs of tracheal elements connecting callus to explant (Maciel et al., 2010). Somatic embryos grew on epidermal and subepidermal tissue of juçara (Guerra and Handro, 1998) and açaí zygotic embryo explants (Scherwinski-Pereira et al., 2012a). Silva et al., (2012) showed that the first signs of cellular division on oil palm zygotic embryos were from the procambial and perivascular cells, theorizing that hydrolysis of lipid reserves in the cotyledon gave perivascular cells a supply of rich energy-dense compounds to fuel cell growth and division. Embryogenic calli or meristematic cells growing from explants have been described as having a dense cytoplasm, a voluminous central nucleus, high nucleoplasmic ratio, and a visible nucleolus (Reynolds and Murashige, 1979; Gueye et al., 2009; Silva et al., 2014). Another important observation is that these cells often have starch reserves (Verdeil et al., 2001), which tended to disappear as calli grew into proembryos (Goh et al., 2001). These cell types have been used in histological markers, and therefore can be considered a marker of embryogenic competence due to their high mitotic activity and energy reserves. As SE progresses, additional, and often more easily recognizable, markers of SE arise as the embryos develop.

Extracellular matrix

A common marker of embryogenic competence for embryogenic calli and proembryos is the formation of an extracellular matrix, a “net” of microfilaments, lipophilic substances, arabinogalactan proteins, and other substances excreted by cells (Samaj et al., 1999). The role of the extracellular matrix within SE might be to guide cell fate to an embryogenic state through reception and transduction of signal molecules from neighboring cells (Palanyandy et al. 2013). Steinmacher et al. (2012) analyzed arabinogalactan proteins and the extracellular matrix of peach palm somatic embryos, noting that areas previously covered by

extracellular matrix tended to promote secondary somatic embryogenesis. The authors observed that the matrix contained lipophilic substances, which might act as signal molecules to other cells, controlling cell fate and their ability to re-differentiate (Kader, 1997). Expressed sequence tags (ESTs) identified from oil palm notably showed lipid transferase proteins (LTP) highly expressed in embryogenic tissue (Low et al., 2008), which was also found in *Elaeis guineensis x E. oleifera* hybrid callus cultures (Angelo et al., 2013). The JIM5 antibody, an indicator of lipid transferase activity, appeared in the middle lamella in coconut cultures, showing that accumulation of polygalacturonic acid at cell wall junctions between cells is possibly involved with SE (Verdeil et al., 2001). In the same report, no cytoplasmic continuity between neighboring cells was observed, suggesting that embryogenic founder cells were isolated from their neighbors. This change in cell-cell communication could also cause metabolic changes, triggering the expression of new genes and, possibly as a result, SE. Although not described as an extracellular matrix, several observations in other papers might describe its formation, such as callus-forming soft glutinous structures (Wang et al., 2003). *Butia eriospatha* embryogenic cultures were recently observed to form a thick, sticky substance suspected to be extracellular matrix. These cultures developed into nodular structures, although somatic embryos have yet to be identified (Unpublished data). Goh et al., (2001) described a "polysaccharidic mucilage" that covered *Calamas merrillii* embryogenic cells of friable yellow calli in active division which gave rise to proembryogenic clumps when transferred to culture media with low auxin levels. While not studied in depth, the presence of an extracellular matrix was observed in macaw palm (*Acrocomia aculeata*) embryogenic cultures (Moura et al., 2010).

Globular embryos and maturation

After SE induction and adequate tissue culture conditions, ordered cell division yields small immature globular embryos. Globular embryos are smooth, round structures containing bilateral root/shoot symmetry, and a defined protoderm. Similar to globular zygotic embryos, globular somatic embryos can have a developed suspensor region that connects the developing embryo to the explant tissue (Scherwinski-Pereira et al., 2010). Moura et al. (2009) showed that globular macaw palm somatic embryos displayed a protoderm, apical meristems, and procambial strands. The protoderm of nodular structures growing out of peach palm

embryogenic calli showed the first and second cell layers had low-density cytoplasm and non-evident nuclei, which is in contrast to deeper cell layers which had dense cytoplasm and prominent nuclei (Maciel et al., 2010). However, at this stage the embryo is still immature and must further develop specialized tissues and accrue reserve compounds during maturation.

During maturation, the morphology of the immature globular embryo changes through elongation of the cotyledon, which is composed of the scutellum, the tissue specialized for absorbing nutrients from the endosperm, and the coleoptile, the protective tissue covering the plumule. Longitudinal sections of maturing date palm somatic embryos showed differentiated vascular systems along the cotyledon (Akshaya and Atul, 2013). In zygotic embryo maturation, the embryo begins to accumulate storage compounds, such as carbohydrates, oils, amino acids, and proteins, such as glutelin and Late Embryogenesis Abundant (LEA) proteins. Each of these compounds provides essential functions toward embryo maturation and germination, which can be as simple as an energy source to fuel cell growth and division or as complex as the role of LEA proteins during late embryo maturation. LEA proteins are thought to act as kinetic stabilizers of aggregating proteins, thereby preventing damage to the embryo triggered by desiccation until the embryo develops into a solid state where aggregation ceases (Liu et al., 2011). Dehydrin proteins, a family of LEA proteins induced through desiccation (Close, 1997), have been detected during the development of *Euterpe edulis* zygotic embryos (Panza et al., 2007). Starch was found to accumulate quickly during oil palm SE induction, but decreased only to accumulate again during maturation (Gomes et al., 2014). In the same study, the total amount of free amino acids remained stable for the first 30 days of culture before greatly increasing in concentration and remained at the same level with some fluctuation; however, total protein concentration remained about equal from early induction to late maturation. Biochemical analysis of developing date palm zygotic embryos showed that starch tended to accumulate early during embryogenesis, with glutelin accumulating during maturation (Sghaier-Hammami et al., 2009a). Metabolism changed from a fermentation strategy to respiration during germination, which relied on stored compounds to promote development and the establishment of autotrophy (Sghaier-Hammami et al., 2009a). Date palm zygotic embryos had overall greater abundance of proteins, a higher

proportion related to storage and stress, as well as glutelin, while their somatic embryo counterparts had less protein, much of which was related to glycolysis (Sghaier-Hammami et al., 2009b). Sghaier et al., (2009) noticed increased storage and stress-related proteins and decreased metabolism-related proteins in date palm somatic embryos in media with abscisic acid (ABA) and increased sucrose. Higher concentrations of the storage protein glutelin were measured in somatic embryos cultured with 20µM or 4µM ABA over untreated control without ABA (Sghaier et al., 2008). Similarly, Sghaier-Hammami et al. (2010), found that date palm cultures treated with 10µM ABA, 20µM ABA, or 90g/L sucrose increased overall protein content. Protein deficit in somatic embryos, compared to zygotic embryos, has been observed in several palm species, such as date palm (Sané et al., 2006), oil palm (Aberlenc-Bertossi et al., 2008), and macaw palm (Moura et al., 2010).

Successful conversion, the process similar to germination in ZE, when the embryo begins the transition to a plantlet, depends on the formation of both the radicle and plumule during early SE and the accumulation of reserve compounds sufficient to reach autotrophic conditions. Plant growth regulators have often been shown not to be essential for converting mature somatic embryos, such as in date palm (Bekheet et al., 2001) and macaw palm (Moura et al., 2009), although this is not a universal observation. Culture medium supplemented with 20µM 6-benzylaminopurine (BAP) enhanced germination of zygotic embryos of areca palm (Karun et al., 2004).

Acclimatization

Lack of cuticle on *in vitro* plants, as observed in peach palm (Batagin-Piotto et al., 2012), is heavily responsible for the rapid dehydration that regenerated plants undergo if taken quickly from the high humidity environment of tissue culture to green-house conditions. Planting in soil and keeping them inside a high humidity environment with gradual humidity reduction over time has been demonstrated to produce acclimatized peach palm plants (Steinmacher et al., 2007a). Hardening for 12 months in culture flasks improved acclimation in date palm (Fki et al., 2013). The high mortality of *Calamus thwaitesii* plantlets during acclimation was reduced by dipping plantlets in 50% glycerin to reduce evapotranspiration (Hemanthakumar et al., 2012). Konan et al. (2007)

claimed that genotype was a major factor in determining successful rooting of oil palm regenerants. Addition of 200µM 1-naphthaleneacetic acid (NAA) to media stimulated root growth from cultured coconut embryos with the consideration that plantlets with better-developed root systems would be easier to acclimate to environmental conditions (Ashburner et al., 1993). Treatment of bottle palm *in vitro* seedling with 0.05mg/L paclobutrazol increased the acclimation success rate (Sarasan et al., 2002). Coconut plantlets produced from zygotic and somatic embryos were compared, finding little difference in physical and morphological characteristics, with the sole exception of slightly altered inflorescences from *in vitro*-derived plantlets (Koffi et al. 2013).

Cryopreservation

Genetic gains from breeding programs, novel mutants for study, or vast collections of wild germplasm may be cryopreserved at ultra-low temperatures, leaving tissue in a state of metabolic inactivity until the samples are revitalized. Palm tissue has been successfully cryopreserved in several species either by directly submerging cryotubes with tissue in liquid nitrogen, as had been performed with oil palm somatic embryos (Dumet et al., 1993), or by using specialized protocols, such as encapsulation (Rai et al., 2009) or vitrification (Heringer et al., 2013a). Encapsulation subjects tissue samples to sodium alginate solution, pipetting the solution with the tissue samples, and placing samples onto calcium chloride. This prevents ice crystal formation, a condition that ruptures cellular walls and leads to cell death. Dehydration encapsulation has been successfully performed in *Calamus thwaitesii* with 66.7% regeneration efficiency of frozen tissue (Hemanthakumar et al. 2012). Another cryopreservation protocol, vitrification, uses high sugar concentration to pretreat tissue, thereby increasing endogenous solute concentrations high enough to prevent ice crystal formation (Engelmann, 2011). Vitrification solutions composed of different levels of high concentration glycerol, sucrose, and, occasionally, DMSO, can also be used to pretreat tissue (Suranthran et al., 2012). Vitrification solutions have been demonstrated to be useful for cryopreservation and revitalization of several palm species, such as oil palm (Surantharan et al., 2012) and both peach palm zygotic (Steinmacher et al.,2007) and somatic embryos (Heringer et al., 2013a). A variation of vitrification is droplet-vitrification, in which tissue is loaded onto foil strips in droplets of vitrification solution and then submerged in liquid nitrogen (Heringer et

al., 2013b) Droplet-vitrification, encapsulation, and standard vitrification, in order of decreasing success, were compared with all three protocols yielding regenerated plants (Fki et al., 2013). Rival et al. (2010) found that seedlings from cryopreserved coconut zygotic embryos did not show altered morphological, cytological, or molecular changes when compared to seedlings from non-cryopreserved zygotic embryos. However, seedlings from cryopreserved zygotic embryos initially grew slower after recovery, but this trend disappeared over time after the plantlets were allowed to grow further.

Explant choice

Tissue collection, endophytes, and decontamination

Before culture initiation can occur, fungal and bacterial contaminates must be removed. If decontamination steps are not taken, contaminants can grow unhindered in the nutrient-rich tissue culture environment and ruin cultures, thus resulting in lost resources, tissue, and time. Explant tissue selection is, therefore, of the first main concerns of palm SE. Teixeira et al., (1994) states that immature oil palm inflorescences are advantageous because they are protected by leaf-like sheaths which prevent infestation by contaminants and protect against sterilization solutions. Antioxidants, such as citric acid and ascorbic acid, have been frequently used to reduce oxidative tissue damage on date palm offshoots (Al-Khayri, 2011a). Ascorbic acid prolongs the health of tissue until it can be sterilized and placed on culture. In addition to contamination from outside sources, endophytic organisms inhabit vascular tissue of many palms, such as in açaí (Rodrigues, 1994), date (Gómez-Vidal et al., 2006), and oil palms (Pinruan et al., 2010). Proteomic analysis of date palm sap identified several proteins potentially from *Saccharomyces cerevisiae* (Thabet et al., 2010). Hardy bacterial or fungal organisms may resist initial cleaning and cause bottlenecks in laboratories.

Commercial bleach solution (2% or greater) with a drop of Tween 20 per 100ml for ten to thirty minutes followed by three or more washes with distilled deionized water is a common protocol for tissue sterilization in peach palm (Steinmacher et al., 2007b), which is similar to decontamination protocols mentioned in many other palm studies. Zulkepli et al. (2011) tested several tissue decontamination protocols for the Malaysian palm, jungle salak (*Salacca glabrescens*) inflorescences. The authors stated that, of the treatments tested, the best decontamination

rate was achieved with the following steps: 15-20 minute wash under running water, soaking in detergent overnight in an incubator shaker, three washes with distilled water, five minute soak in 1% benlate®, wash with sterile water, and, under aseptic conditions, soak the inflorescences in 70% ethanol for 30 seconds and then 20% commercial bleach solution with tween20 for 15-20 minutes, followed by a final rinse with distilled water before culturing the explants on medium. Quickly dipping excised date palm tips in 70% ethanol and surface sterilizing by flaming was used by Kurup et al., (2014) as a precaution against contamination before explants were divided for SE induction.

Zygotic embryos

Zygotic embryos were the most common explant source of all reviewed studies, with exception for date palm studies, which favored using shoot meristems from offshoots. For a list of the most commonly used explant source and their associated advantages/disadvantages, see Table 1-2. Despite the drawback of unknown genetic makeup due to sexual recombination, zygotic embryos provide responsive tissue with little to no damage to the mother plant. However, zygotic embryo maturity affects culture initiation. Teixeira et al. (1993) showed that immature zygotic embryos tended to make embryogenic calli at higher rates than mature zygotic embryos, which tended to form organogenic growths and non-embryogenic callus. Separated zygotic embryo tissues, such as cotyledonary nodes have been separated and effectively used as explants, such as in oil palm (Jayanthi et al., 2011). Chan et al. (1998) reported nearly double calli growth and ten-fold somatic embryo growth on mature coconut zygotic embryo plumules in comparison to inflorescence explants. Through a combination of multiplication of embryogenic calluses and secondary SE, the total yield of somatic embryos per induced plumule increased thousands of times more, thus showing that plumule cultures are responsive to SE and can be successively multiplied to mass-propagate a single individual plant (Pérez-Núñez et al., 2006).

Meristems, thin cell layer, and leaf tissue

Meristem tissue is a desirable explant source because, like zygotic embryos, the tissue tends to respond quickly *in vitro*, yielding vigorously-growing cultures. The major drawback, however, is that shoot meristems are often few in number for many of the most common palm species. In the case of single-growing apex palms, such as juçara (Saldanha and Martins-Corder, 2012), harvesting shoot meristem tissue kills the plant, making shoot meristem explants highly destructive to adult plant populations. Date palm SE protocols have made frequent use of shoot meristems harvested from offshoots of adult trees (El-Hadrami et al., 1995, Veramendi and Navarro, 1997, Al-Khayri, 2011a). However, meristematic tissue from young plantlets has high embryogenic potential as shown through the use of the thin cell layer (TCL) technique for peach palm (Steinmacher et al., 2007c), macaw palm (Padilha 2013), and oil palm (Scherwinski-Pereira et al., 2012b). TCL, first described by Tran Than Van, (1973) in tobacco, uses 1mm or thinner tissue segments as explants. Studies using peach palm plantlets (Steinmacher et al., 2007c), oil palm plantlets (Scherwinski-Pereira et al., 2010), and macaw palm microplants (Padilha, 2013), found that segments excised at the apical meristem were the most responsive to culture with reduced vigor from segments excised either below and above the meristem. While not necessarily referred to as TCL, Karun et al., (2004) sliced areca palm inflorescences into 2mm segments with successful tissue regrowth. While more destructive than zygotic embryos as an explant source, meristems and TCL provide means of reliably regenerating embryogenic tissue. Leaf tissue, although abundant, has comparatively lacked the regenerative potential of zygotic embryos or meristematic tissue. Furthermore, protocols might not have yet been fully optimized to use leaf tissue. Almeida and Almeida (2006) demonstrated a protocol in which leaves were placed on media with the cytokinin BAP, rather than on media with high auxin concentrations usual for SE protocols. The epidermal tissues of these leaves oxidized, but the mesophyll cells inside the leaves remained metabolically active and began dividing. Embryogenic callus burst through the oxidized epidermis and gave rise to somatic embryos. Fki et al. (2011) likewise found that the superficial cell layers in date palm leaves had high callogenic capacity. El-Kazzaz and El-Bahr, (2001) observed callus proliferation on leaf middle ribs in date palm cultivar 'Samany' cultures. *In vitro* techniques were developed for sealing wax

palm, finding that young leaves were the least prone to browning (Marzuki, 1997). Despite producing fewer somatic embryos than shoot tip explants, leaflet segments were successfully used as explants in date palm (Ibraheem et al., 2013). Reliable use of mature palm leaves has not been demonstrated yet, but young leaf segments might provide an abundant explant source without the drawbacks of unknown genotype from zygotic embryos, not the destructiveness of meristem and immature leaf harvest.

Inflorescences and other explant sources

Inflorescences have been as explant sources in juçara (Guerra and Handro, 1998), coconut (Verdeil et al., 1994), areca palm (Karun et al., 2004), date palm (Abul-Soad and Mahdi, 2010) and oil palm (Teixeira et al., 1994; Guedes et al., 2011), but this explant source was not as widely used as zygotic embryos or meristems. Commercial propagation has been achieved for date palm according to Abul-Soad and Madi (2010), who reported that inflorescence-based SE protocols have yielded over 10,000 regenerated plants from a single explant that had been multiplied. Other tissue sources have also been used for explant sources in palm protocols. Peach palm staminated flower buds were used as an explant source that, despite intense browning, regenerated "buds" after ten months, which proceeded to multiply when separated from the explant (Almeida and Kerbauy, 1996). Germ-pores containing ovule sections were excised from date palm flowers to create calli (Reynolds and Murashige, 1979). Unfertilized embryos were used to create coconut cultures (Pereira et al., 2009). Sukamoto (2011) used coconut endosperm cylinders to initiate cultures.

Table 1-2: The most common explant source tissues and their advantages and disadvantages

Explant source	Advantages	Disadvantages
Zygotic embryos	• Highly responsive • Abundant in many species • Little to no destruction of existing plant	• Unknown genotypes, unless successfully selfed • Some species produce few seeds • Age of zygotic embryo affects culture response
Shoot tips	• Highly responsive • Ability to propagate a single individual reliably • Relatively easy creation of embryogenic cell lines	• Destructive to existing plant • Limited supply • Some species have only one growing apex, harvest sacrifices entire trunk
Young leaves	• Plentiful tissue • Propagates a single individual • Low destruction to existing plant	• Lacks regenerative potential in comparison to zygotic embryos and shoot tips • Comparatively few studies have used developed leaves
Inflorescence	• Only mildly destructive • Propagates single individual • Successful protocols in many species	• Lacks regenerative potential in comparison to zygotic embryos and shoot tips
Flowers	• Little destruction • Propagates single individual • Depending on the palm species, tissue is plentiful	• Limited success in comparison to other types of tissue • Very few protocols exist
Roots	• Multiple meristems present • Some palm species have many branching roots and, therefore, more root meristems	• Decontamination difficult • Very few protocols • Limited success in comparison to other types of tissue • Moderately destructive

Protocol improvements

Optimization studies have revealed new strategies to improve palm SE. A selection of palm SE optimization studies have had their SE protocols and key observations summarized in Table 3.

Solid vs. Liquid Media

Liquid media and media solidified with gelling agents present two vastly different tissue culture environments. The advantages of liquid media are the lack of nutrient gradients and access of more tissue surface area to nutrients, while the main advantage of solid culture is gas exchange. Date

palm SE protocols extensively use liquid culture in one or more steps, especially to create suspension cell cultures. Indeed, for date palm, liquid culture has shown to promote SE more than solely using solid cultures, such as after callus induction to increase somatic embryo population over solid media (Fki et al., 2003; Zouine et al., 2005). Bhaskaran and Smith (1992) claimed that date palm liquid cultures could produce hundreds of somatic embryos from only a small amount of tissue. In comparison to solid media, maturation was improved using liquid media over solid media in Canary Island date palm (Huong et al., 1999). Suspension cultures have had success for both oil palm (Touchet et al., 1991; Kramut and Te-chato, 2010) and date palm (Abohatem et al., 2011). Oil palm suspension cell cultures from friable embryogeneic calli were plated onto a regeneration media, which developed into mature embryos and successfully converted into plantlets (Teixeira et al., 1995). Similar to bacterial growth curves, the lag, exponential, linear, and stationary phases have been identified in date palm suspension cultures (Al-Khayri, 2012). Inoculum size in liquid cultures have been tested in oil palm (Gorret et al., 2004) with the finding that higher inoculum density and media conditioned for two weeks in other embryogenic cultures before reuse in new cultures have a positive effect on biomass production. Badawy et al. (2009) observed increased somatic embryogenesis using 500µM and 200µM straining meshes as compared to 100µM mesh, thus showing that larger inoculum size improves somatic embryogenesis. Temporary immersion systems, a variation of liquid culture systems, present advantages over both full immersion systems and solid media, such as greater gas exchange and lack of nutrient gradients, respectively. A temporary immersion system yielded high numbers of regenerated embryos in peach palm over solid cultures (Steinmacher et al., 2011). However, tissue culture environment might be needed to be optimized for each genotype. In a comparison between solid, liquid, and temporary immersion systems on oil palm SE, Ibraheem et al., (2013) reported that far greater numbers of somatic embryos grew in liquid media than on either solid media or in a temporary media system.

A promising means of culture multiplication is the use of protoplasts. In palms, there are comparatively few protoplast isolation and culturing protocols, but successful regeneration has been reported. Oil palm protoplasts were isolated and induced to multiply into microcalli (Bass and Hughes, 1984; Sambanthamurthi et al., 1996). Protoplasts were

isolated from young leaf and embryogenic calli cells of 'Deglet nour' and 'Takerboucht' date palms and these protoplasts divided to grow microcalli, but further development into somatic embryos was low (Chabane et al., 2007). Through careful monitoring and media optimization Masani et al., (2013) successfully induced oil palm protoplasts from suspension cell cultures to grow into microcalli, which then further developed into embryogenic callus, somatic embryos, and then converted into complete plantlets.

Carbohydrate source

With some exceptions, most of the reviewed studies used 30g/L sucrose as a carbohydrate source for all media. Modifying carbon source concentration has been a focus of study in palm cultures, although with the trend that 3% sucrose, more often than not, was the optimal concentration for SE, as shown in date palm (Bekheet et al., 2001; Taha et al., 2001, Asemota et al., 2007). When compared, glucose was outperformed by sucrose in juçara leaf sheath explants (Saldanha and Martins-Corder, 2012). In a comparison between sucrose concentrations on date palm SE, 3% had the best results in several genotypes, although at the cost of increased phenolic content (Zouine and El Hadrami, 2004). Complete lack of carbon sources resulted in failed regeneration, thus demonstrating that carbohydrates have a crucial role in fueling SE and regeneration (Alkhateeb, 2008). Date palm liquid media cultures starved of sucrose for two weeks and then cultured on 3% sucrose media showed enhanced embryo formation over cultured continuously kept in 3% sucrose media (Veramendi and Navarro, 1996). Sorbitol performed equal to or better than sucrose, glucose, fructose, and mannitol in oil palm cultures at 0.1M sugar concentration on full MS, but 0.3M sucrose and glucose had better results than 0.3M sorbitol (Te-Chato and Hilae, 2007). In later work, Chehmalee and Te-chato (2008) reported using 0.2M sorbitol to induce secondary SE. Gorret et al., (2004) showed that sucrose broke down rapidly into glucose and fructose in an oil palm liquid bioreactor system.

Auxins

SE induction requires exogenous auxins, such as 2,4-D, picloram, or dicamba in order to induce culture growth, as demonstrated in sago palm (*Metroxylon sagu*) (Alang and Krishnapillay, 1987), coconut (Karunaratne and Periyapperuma, 1989), açaí (Ledo et al., 2002), areca palm (Wang et al., 2003, Wang et al., 2006), peach palm (Steinmacher et al., 2007b), and oil palm (Guedes et al., 2011). Auxins, with or without other growth regulators, influence culture activity (for review, see Jiménez 2005). Interestingly, juçara cultures have been observed to produce anthocyanin in response to either 2,4-D (Guerra and Handro, 1998) or picloram (Saldanha and Martins-Corder, 2012). Comparison studies of 2,4-D and picloram tended to show improved regeneration in picloram cultures of areca palms (Karun et al., 2004), peach palm (Steinmacher et al., 2007), oil palm (Scherwinski-Pereira et al., 2010), and açaí (Scherwinski-Pereira et al., 2011), but the opposite has also been reported in oil palm (Thuzar et al., 2011). However, auxin comparisons, such as the effect of picloram vs. 2,4-D in macaw palm (Moura et al., 2007), showed that both auxins are capable of inducing SE. TCL sections from macaw palm microplants regenerated callus efficiently on media supplied with 150µM to 600µM picloram with greatest growth of nodular calli at 150µM picloram (Padilha et al., 2013). In a comparison between 2,4-D and NAA, Alang and Krishnapillay (1987) showed that high 2,4-D in the presence of activated charcoal (AC) was essential for SE induction. 2,4-D was preferable to NAA for SE induction in date palm cultures (Sané et al., 2012). Curiously, auxin concentration can affect what kind of tissue is regenerated, as shown in date palm by Gueye et al. (2009), who observed that low concentrations (1µM) of NAA promoted root growth from cultured leaf explants, while higher concentrations (54µM) of NAA promoted callus growth.

After growth of embryos or embryogenic calli, subculturing of explants onto media with either reduced or completely eliminated auxin concentrations is a common step used to promote embryo development. Reynolds and Murashige (1979) described embryos developing on excised ovule date palm explants after reculturing on auxin-less media. Indeed, lower concentration or complete removal of auxin has been used successfully in coconut (Verdeil, 1994), two rattan palms (Goh et al., 1999), saw palmetto (*Serenoa repens*) (Meagher and Green 2002), and areca palm (Karun, et al 2004) which yielded embryos that can either be further matured or converted into plantlets. Replacing picloram or 2,4-D

with low NAA concentrations has been used in maturation stage during SE, as shown in oil palm (Teixeira et al., 1993), juçara (Guerra and Handro, 1998), and açaí (Scherwinski-Pereira et al., 2012).

Activated charcoal and antioxidants

AC has been included in the SE protocols of several palms, especially in the initial establishment phase. It's inclusion in media has been reported essential in sago palm (Alang and Krishnapillay, 1987), juçara (Guerra and Handro, 1998), and macaw palm (Moura et al., 2008). This is not a universal observation, as shown in Canary Island date palm where addition of activated charcoal (AC) resulted in callogenic tissue with diminished embryogenic potential compared to cultures without AC (Huong et al., 1999). Balzon et al. (2013) reported that they were able to regenerate embryogenic oil palm calli with or without AC in culture medium with picloram, although optimum results were obtained with AC. While not necessarily essential, charcoal greatly improved growth in oil palm thin cell layer inflorescence explants (Guedes et al., 2011) and zygotic embryos (Suranthran et al., 2011). In both studies, high concentrations of 2,4-D were required when AC was added in order to stimulate culture growth. AC can have far-reaching effects on *in vitro* cultures (for review, see (Pan and Staden, 1998) such as the need for increased concentration of auxin necessary to have the same effect in media without AC. In a review Weatherhead et al., (1978) suggested that the effect of charcoal in tissue culture systems is dependent on tissue, species and genotype. The effects might be attributed to a darkened environment, absorption of undesirable substances, absorption of growth regulators, or the release of growth-promoting substances by charcoal. The type of charcoal used can have different effects on cultures, as demonstrated by Sáenz et al., (2010), who used eight types of charcoal to test the level of 2,4-D, pH, conductivity, osmolarity of the culture medium, and callus induction frequency and found some slight, but occasionally significant, variation between each charcoal type.

AC reduces the oxidative stress of reactive oxygen species (ROS) (Apel and Hirt, 2004). ROS, in the form of hydrogen peroxide and superoxide, are both produced through several common enzymes, such as xanthine oxidase, lipoxygenases, peroxidases, NADPH oxidase, and canthine oxidase (Blokhina et al., 2003). High levels of ROS in the cell may

denature proteins, cause the peroxidation of unsaturated fatty acids, or damage to nucleic acid (for review, see Blokhina et al., 2003). Other chemicals, such as phenolics, tannins, and oxidized polyphenols are synthesized through shikimic acid phenylopropanoids, flavonoid, and terpenoid pathways, potentially leading to inhibitory effects on cellular growth and division (Sukamto, 2011). Addition of high concentrations of both ascorbic acid and citric acid during offshoot apex excision to reduce damage caused by tissue oxidation is a common practice for date palm (Al-Khayri, 2001). However, AC and ascorbic acid were not effective in the establishment of *Calamas nagbetti* cultures; problematic browning caused high explant mortality (Kumar et al., 2009). However, Marzuki et al., (1997) found that addition of antioxidants PVP, ascorbic acid, and citric acid prevented oxidation in sealing wax palm cultures (*Crytostachys renda*).

Cytokinins

Cytokinins, thought to be instrumental in establishing bipolar meristem axes in embryos, have shown a general trend of improving embryo development after SE induction. For example, the addition of 0.25mg/L BAP with 0.5mg/L NAA led to significantly higher numbers of somatic embryos on callus cultures than cultures with 0.5mg/L NAA alone in date palm (Kurup et al., 2014). While an "auxin shock" has been applied in many protocols, Almeida and Almeida (2006) demonstrated that BAP alone was enough to induce peach palm embryogenic calli and somatic embryos using leaf explants. Low levels of BAP (0.3mg/L) improved date palm SE in suspension cultures, whereas increased concentrations of 0.4mg/L and 0.5mg/L decreased total number of embryogenic masses (Abohatem et al., 2011). The type of cytokinin can have a dramatic effect on culture development. BAP had either no effect or detrimental effect in date palm cultures, but inclusion of 2-isopentenyladenine (2-iP) improved the formation of shoot buds (Taha et al, 2001). Many cytokinins, such as 2ip, BAP, kinetin, and zeatin, are derived from adenine, the sole exception being thidiazuron (TDZ). Adenine sulfate has been tested as an improvement to date palm SE protocols, with some success in promoting date primary callus growth (Sané et al., 2012). A combination of 2-iP, Kinetin, and BAP (1mg/L each) and 0.5mg/L of the auxin naphthoxy acetic acid (NOA) led to increased fresh weight and axillary bud

proliferation in date palm (Hegazy and Aboshama, 2010). Cytokinins were observed to be effective at increasing conversion of oil palm embryos by as much as 70% by boosting shoot apical meristem formation (Aberlenc-Bertossi et al., 1999). After SE induction, addition of BAP was instrumental in the complete differentiation of coconut embryos with a root meristem and a single shoot meristem (Verdeil, 1994). Perera et al. (2009) likewise also observed improved coconut shoot regeneration using 2-iP. Interestingly, TDZ pulses during rooting of peach palm plantlets resulted in the formation of prickles along the epidermis of regenerated plants, in contrast to control, which grew none (Graner et al., 2013).

Basal media

The most common overall basal media used, with the possible exception of coconut studies, was MS media (Murashige and Skoog, 1962). Several comparison experiments have been done to optimize palm protocols, most commonly comparing MS to Eeuwens (Y3) media (Eeuwens, 1976), which was developed for coconut cultures. Callus induction was improved in TCL explants derived from macaw palm microplants on Y3 over cultures on MS media (Padilha et al, 2013). Likewise, jungle salak immature inflorescence explants and coconut zygotic embryo explants regenerated better on Y3 media over MS (Gupta et al., 1984; Zulkepli et al. 2011). However, there were no significant differences when compared in juçara cultures (Guerra and Handro 1998). MS outperformed WPM (Lloyd and McCown, 1980) and NM (Nitsch and Nitsch, 1969) media on date palm cultures (Mazri 2013). When thin cell layer sections of female oil palm inflorescences were cultures on either MS or Y3 media, nodular calli grew significantly more often than on cultures on Y3 media (Guedes et al., 2011). Genotype variation led to different basal salt preferences in date palm (Al-Khayri, 2011). Full MS was often observed to be preferential to half MS in date palm (Taha et al., 2001) and oil palm (Hilae and Te Chato, 2005). In one experiment, oil palm zygotic embryos grew higher rates of embryogenic calli on N6 media (Chu et al., 1975) over MS, with a following experiment finding higher plantlet regeneration on a modified version of N6 media over MS (Thuzar et al., 2011).

Vitamins, amino acids, casein, and yeast extract

MS (Murashige and Skoog, 1962) or MW vitamins (Morel and Wetmore, 1951) were the most used vitamins in reviewed studies. Several groups

have looked to optimize their protocols by adding additional thiamine, pyridoxine HCl, nicotinic acid, and biotin. Badawy et al. (2009) observed that increasing pyridoxine, thiamine, and nicotinic acid concentrations was effective in promoting SE in date palm, but increasing biotin was not as effective. This is in contrast with Al-Khayri (2001) who reported increasing fresh weight and number of embryos on date palm cultures in response to the addition of up to 2mg/L biotin and 0.5mg/L thiamine to the culture medium.

Inclusion of glutamine has been common in many palm SE protocols (Steinmacher et al., 2007a). However, its effect is often not a limiting factor in somatic embryo growth, as demonstrated in juçara by Saldanha et al. (2013), who observed little variation among cultures with differing levels of glutamine. Gorret et al. (2004) did not find glutamine to have a significant effect on the growth of oil palm cultures in a bioreactor, although additional ammonia had a positive effect. Arginine (3mM), was reported to have beneficial effects in date palm (Sghaier et al., 2009). Casein hydrolysate and yeast extract were tested in date cultures, yielding double or more callus weight in either treatments, as well as improved number of somatic embryos per callus compared to control (Al-Khayri, 2011b). Casein hydrolysate also improved protocols, as demonstrated by Khierallah and Hussein (2013), who observed increased callus formation, somatic embryo growth, and conversion with 2g/L casein, the highest amount tested, in date palm cultures.

Silver nitrate

Silver nitrate, an effective ethylene antagonist, had been tested on several palms with variable results based on species and genotype. A review by Kumar et al., (2009) covers silver nitrate as a potential growth modulator. The silver ion mediates responses involved with polyamine synthesis, ethylene, and calcium-mediated pathways, resulting in a range of modifications to cellular conditions and activity. Due to the wide array of functions that ethylene plays in tissue culture (for review, see Biddington, 1992), it is difficult to predict the effect of exogenous silver nitrate. Addition of silver nitrate to the culture medium improved the number of regenerated embryos in four of five date palm genotypes, but each cultivar had its own optimum concentration (Al-Khayri, 2003). In previous work, (Al-Khayri and Al-Bahrany, 2001) observed an increased number of

somatic embryos in response to increasing levels of silver nitrate, but this trend was reversed with the addition of increasing 2-iP with silver nitrate, with optimal number of regenerated embryos growing on MS media with 25µM silver nitrate and 0.5µM 2-Ip. Silver nitrate, however, had a negative effect in macaw palm SE (Padilha et al., 2013).

Gibberellic acid

Gibberellins create wide shifts in protein expression and abundance, thus, creating large-scale changes in gene expression, cell behavior, and metabolism (Hochholdinger et al., 2006). The addition of 1mg/L gibberellic acid (GA) to the culture medium reduced browning at the expense of increased callusing in cultures of bottle palm (Sarasan et al., 2002). Coconut somatic embryo conversion into plantlets was improved in response to the addition of GA and BAP to the culture medium (Perera et al., 2009). Additionally the supplementation of GA to the culture medium increased the number of coconut calli that formed somatic embryos by 50%, nearly doubling the number of somatic embryos per callus, causing earlier expression of a coconut KNOX1 gene, and decreasing the expression of the KNOX2 gene (Montero-Cortes et al., 2010).

ABA

ABA, one of the most common naturally-occurring plant hormones stimulates transcription of numerous genes, prevents precocious germination, and stimulates embryo maturation (for review, see Rai et al., 2011). ABA shows variable effects on SE in palms. Increased numbers of somatic embryos were recovered from date palm cultures in response to exogenous ABA (Zouine et al., 2005), as well as increased protein levels, an observation also made by Sghaier-Hammami et al., (2010). Additionally, ABA increased the thickness of somatic embryos, increased their dry weight, and promoted embryo proliferation in date palm cultures (Sghaier et al., 2009). However, addition of ABA to coconut cultures stunted culture growth and, later, shoot regeneration (Perera et al., 2009). Fernando and Gamage (2000) reported improved SE in coconut using exogenous ABA, but did notice a distinct failure of shoot regeneration at the highest concentration of ABA used. Overall, expanded ABA optimization in palm tissue culture might be beneficial for improving

successful conversion of somatic embryos into plantlets through increased reserve compounds and improved maturation.

Polyamines

Polyamines are an essential group of organic compounds with two or more amino groups found in both eukaryotes and prokaryotes (Kaur-Sawhney et al., 2003). The three most well-studied polyamine molecules in plants, spermine, spermidine, and putrescine, have functions in several major processes, such as cell division, organogenesis, and stress tolerance. Exogenous polyamines have demonstrated improvements in SE of several palm species, such as oil palm (Rajesh et al., 2003) and date palm (Hegazy and Aboshama, 2010). Rajesh et al. (2003) found that addition of putrescine in the culture medium improved embryogenic calli formation, number of somatic embryos, secondary SE, shoot count, and conversion. Polyamines, however, do not guarantee improved SE in all plants. Noceda et al., (2009) observed that decreased free polyamines levels, as well as DNA demethylation, were associated with SE in *Pinus nigra*. Engineered accumulation of spermine and spermidine in tomato restored metabolic activity in late stages of fruit ripening by reopening biochemical pathways related to N:C signaling, energy, and glucose metabolism (Mattoo and Handa, 2008). These studies show that polyamines have complex and variable effects on cellular activity. More studies on exogenous polyamines are necessary before suggesting their use for standard protocols.

Coconut water and date syrup

The additives coconut water and date palm syrup have shown mostly positive responses. Al-Khayri, (2010) observed that callus fresh weight and SE induction increased with addition of 10% and 15% coconut water to MS media for date palm cultivars 'Khasab' and 'Nabout Saif', respectively. Media composed of 20% coconut water more than doubled the number of somatic embryos produced in cultures of the date palm cultivar 'Bream' compared to media without coconut water (Khierallah and Hussein, 2013). Likewise, Patcharapisutsin and Kanchanapoom (1994) included coconut water as part of maturation media with NAA or 2,4-D to enhance mature oil palm embryos. Addition of coconut water to the culture medium can provide improved results in non-palm plants, as in the case of production of vigorous *Dendrobium alya* plantlets in

response to 10% coconut water (Nambiar et al., 2012). Date palm syrup, a less-expensive possible alternative to sucrose, was compared as a carbon source to sucrose in date cultures. Compared to media supplemented with 4, 6, and 8% date syrup, media supplemented with 3% and 6% sucrose regenerated more embryos (Alkhateeb, 2008). However, treatments with date syrup did regenerate embryos, which show the possible use of cheaper alternatives to sucrose as a viable option for limiting laboratory expenses.

Lightly-covered topics

Chopping date palm calli led to actively growing friable structures composed of cells with small vacuoles and high concentrations of soluble proteins, characteristics similar to embryogenic cells (Sané et al., 2006). Othmani et al. (2009) used partial desiccation over several time intervals and two methods of fine chopping to vastly increase the number of somatic embryos produced by date palm calli in a liquid culture system. Six-hour proembryo desiccation more than doubled the average number of somatic embryos regenerated, while a 12-hour desiccation increased it nearly six-fold. Likewise, chopping with a scalpel or straining the calli through a mesh with a glass pestle generated about eight times the number of somatic embryos than control methods (Othmani et al., 2009). In some studies, cultures cultivated in either the light or dark grew with little difference between the two (Bhaskaran and Smith, 1992). When placed in the light, coconut cultures grew nearly seven times the number of calli bearing embryos than cultures maintained in the dark (Chan et al., 1998). Date palm cultures in liquid MS media benefited from seven-day subculture intervals over longer intervals, yielding more embryogenic cells with less oxidation damage (Abohatem et al., 2011).

Future Research

SE in palms has been successfully induced with a similar pattern. Zygotic embryos or shoot meristems have had the overall greatest success if induced on MS or Y3 media supplemented with AC, sucrose, and 2,4-D or picloram. However, several major obstacles remain to be overcome. First, zygotic embryos and shoot tips, although highly responsive and relatively reliable, have major drawbacks. Improved SE induction on other explant source tissues, such as young leaves and inflorescences,

would allow a wider range of genotypes to be collected from the field with minimal damage to existing plants. Second, genotype-linked recalcitrance to SE can hinder use of select genotypes, including elite cultivars. The genetic and biochemical mechanisms of recalcitrance should be studied and then used to manipulate existing SE protocols to suite a wider range of genotypes, thus strengthening the role of SE in agriculture, science, and conservation. Third, poor somatic embryo maturation might be the major factor in the low conversion rates reported in many palms. Improved maturation steps leading to increased nutrient reserves might lead to increased conversion rates and overall regeneration efficiency. Fourth, 'mantled' oil palm phenotypes wreak a toll on overall yield and wastes farmer resources. Early detection of somaclonal variation could not only benefit oil palm propagation, but might be an important diagnostic that can be applied to other palms. Finally the emergence of the ´omics´ brings new strategies to advance our understanding of palm SE. These can be applied to better understand ZE and find methods of modifying SE protocols so that regenerated somatic embryos better match the biochemical states of zygotic embryos. Taken together, an ideal goal would be to take a an inflorescence or leaf clipping from any given palm, induce SE, complete maturation, convert embryos, and have regenerated plantlets complete their life cycles and bear fruit without the debilitating results of somaclonal variation and methylation. Though it is a quite complex goal, success would allow efficient propagation, conservation, reforestation, and improved agriculture output from palm crops.

Concluding remarks

Palm SE studies have uncovered new insights into the mechanics of embryogenesis and new means of optimizing SE efficiency and increased the number of available genotypes for use. The importance of auxin type and concentration has been a main focus of study in many species, but their inclusion in media was often essential for SE induction. Embryogenesis genes, such as SERK, have been identified in several palm studies, often in accordance with results in other species. The complex process of somatic embryo maturation has been analyzed in part with transcriptomics and proteomics, through which the role of ABA showed its integral part in maturation. The mounting number of optimization studies has supported the use of additional compounds beyond basal media, growth regulators, and sucrose to improve palm SE.

The role that palm trees play in modern agriculture will make expanded use of SE protocols for new species and genotypes essential for meet future needs as a technology that will not only preserve genetic gains from breeding programs, but store genetic diversity of wild palm populations. Through continued study of SE mechanics and validating results, especially from studies with significantly great improvements, the goal of being able to take any given tissue from any plant and reliably regenerating somatic embryos will be that much closer to fruition.

Acknowledgements
The authors wish to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES – Brasília/Brazil and the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq – Brasília/Brazil, for financial support.

Table 1-3: Protocol, explant types, media composition and key results and observations from selected palm somatic embryogenesis (SE) papers

Reference	Type of palm	Explant	Media	Key results and observations
Karun et al. (2004)	Areca palm	Young leaves Immature inflorescences	• Callus induction: MS[1], 30g[2] sucrose, 1g AC, 6g agar o Experiment: 68µM 2,4-D, 25, 50µM dicamba, or 100, 200µM picloram • Differentiation/maturation: Same basic media as the first media for this reference (SBM[3]) without auxin and with 2g AC • Conversion: same SBM without auxin o Comparison: 0, 5, 10, 20, 30, 40µM BAP o Comparison: 20µM BAP, Kin, 2iP, or TDZ • Secondary conversion: liquid SBM (liquid) with 5µM BAP	• Both types of explants from all genotypes responded better to picloram • Transfer from high to low auxin was essential for SE induction • Full somatic embryo differentiation was most effective in culture media free of plant growth regulators (PGR[4]) • Somatic embryo conversion was highest on media with 20µM BAP
Wang et al. (2006)	Areca palm	Leaf Root Stem	• Induction: MS, 100mg *myo*-inositol, 0.5mg niacin, 0.5mg pyridoxine-HCl, 0.1mg thiamine, 2mg glycine, 1 g peptone, 170mg NaH2PO4, 30g sucrose, 2.2g gelrite, dark conditions o Comparison: 2, 4, 6, or 8mg dicamba or 2,4-D • Maturation: SBM free of PGR, light conditions	• Auxin was required for inducing callus formation • Both types of auxin were able to stimulate callus growth • Roots, leaves, and then stem explants in order of overall callusing efficiency • Plants regenerated from somatic embryos were successfully acclimatized to greenhouse conditions
Huong et al. (1999)	Canary Island Date Palm	Zygotic embryos Shoot tips	• Callus Induction: MS, 30g sucrose, 7.5g agar o Comparison: nine treatments 2,4-D 4.25-452.5µM with 2ip 0.98-14.7µM or picloram 25-200µM with 2.32, 4.6, or 9.5µM kinetin and 3g AC for highest 2,4D and picloram sets • Multiplication: SBM • SE induction/Maturation: liquid SBM o 0, 1, 2, 3 µM 2,4-D with or without 1µM kinetin, and with 0, 1, 2µM ABA • Conversion: Liquid SBM o Comparison: 0.45, 1, 1.5, or 2µM of BAP, 2iP, kinetin, NAA and IAA • Secondary conversion media: SBM free of PGR	• Zygotic embryos had higher embryogenic potential that shoot tips • Highest concentrations of 2,4-D completely inhibited production of embryogenic callus • Highest callus induction in media supplemented with 10.µM 2,4-D and 2.46µM 2iP, or 100µM picloram and 9.5µM kinetin • Addition of 2µM ABA improved embryo maturation • Liquid media superior to solid media for somatic embryo maturation • Plants were regenerated, but at a low rate
Chan et al. (1998)	Coconut	Zygotic embryo plumules	• Induction: Y3, 3g gelrite, 2.5g AC, dark o Comparison: 0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1mM 2,4-D • Maturation/conversion: SBM with 1µM 2,4-D, 50µM BAP o Comparison: light and dark conditions	• Zygotic embryo germination was commonly observed at the lowest 2,4-D concentrations with increasing rates of callusing up to 0.1mM 2,4-D and necrosis at higher concentrations • Lack of subculture produced a higher proportion of explants developing calli

				• Light conditions improved both the number of calli bearing embryogenic structures and number of calli bearing embryos • Plants were successfully regenerated and acclimatized
Montero-Cortes (2010)	Coconut	Zygotic embryo	• Callus Induction/multiplication: Y3, 2.5g AC, 3g gelrite, 600µM 2,4-D, dark conditions • SE induction/maturation/conversion: SBM with 6µM 2,4-D, and 300µM BA, light conditions ○ Comparison: 0.5µM - 20µM GA	• 0.5µM GA stimulated ~1.5 times the number of calli forming somatic emmryos compared to control and 2 times the number of SE per callus • Two homeobox genes, CnKNOX1 and CnKNOX2 identified to be influenced by GA
Verdeil et al. (1994)	Coconut	Immature inflorescences	• Callus Induction: Y3, MW vitamins, 16.8mM sucrose, 2g AC, 7.5g agar ○ Comparison: 150, 200, 250, 300, 350, 400 µM 2,4-D • SE Induction: SBM with double MS and 87.6mM sucrose ○ 150, 200, 300, 400, 450, 500 µM 2,4-D with gradual reduction over time • Maturation: same as SE induction with reduced or no 2,4-D and 10 µM BAP • Germination: Same as SE induction without PGR	• 200 and 300µM 2,4-D were the most effective for callus formation • Transfer to lower 2,4-D concentrations improved SE maturation • High concentrations of proteins were found in callus but disappeared during proembryo formation • Decreased in 2,4-D and addition of BAP was essential for complete bipolar embryo differentiation
Al-Khayri and Al-Bahrany (2001)	Date palm	Shoot tip	• Primary callus induction: MS salts, 170mg NaH_2PO_4, 125mg *myo*-inositol, 200mg glutamine, 5mg thiamine-HCl, 1mg nicotinic acid, 1mg pyridoxine-HCl, 30g sucrose, 7g agar, 100mg 2,4-D, 3mg 2ip, 1.5g AC, dark conditions • Secondary callus induction: SBM with 53.7µM NAA and 147µM 2iP, light conditions • Embryogenic callus proliferation: SBM with 29.6µM 2iP • Embryogenic callus maintenance media: SBM with 7.4µM 2iP ○ Comparison: 0, 25, 40, 75, 100µM silver nitrate with 0 or 0.5µM 2iP, light conditions • Embryo conversion: SBM with 0.54 NAA, light conditions	• The number of embryos increased with increasing silver nitrate concentrations up to 75µM without 2iP, but the opposite trend was seen when 2ip was added. • The highest number of embryos was counted on media supplied with 25µM silver nitrate and 0.5µM 2iP
Sane et al. (2011)	Date palm (Four different cultivars)	Shoots Roots	• Primary callus induction: MS salts, MW vitamins, 0.01mg biotin, 100mg sodium ascorbate, 100mg *myo*-inositol, 30g sucrose, 8g agar, dark conditions ○ Comparison: 1, 2, 4, 8, 16mg 2,4-D or 2, 4mg NAA with 0, 1mg BAP or 40mg adenine sulfate • Secondary callus induction: SBM with 2mg 2,4-D • Suspension culture establishment: liquid SBM, light conditions • Embryogenic suspension culture: SBM with 20g glucose	• Major genotype-dependent responses were observed with two cultivars producing callus on media supplied with 2,4-D in all treatments and two cultivars barely producing any callus regardless of treatment • 2,4-D was far more effective than NAA for callus induction • Both adenine sulphate and BAP improved callus induction • Highest suspension growth rate was seen with 2mg 2,4D

			○ Comparison: 2mg 2,4-D without AC and 50, 75, 100mg 2,4-D with 1g AC • SE induction: liquid SBM free of PGR • Secondary SE induction: SBM with 60g sucrose ○ Comparison: 0, 0.5, 1, 1.5, 2mg BAP • Conversion: MS with or without 1mg NAA	• A seven-day treatment with 0.5mg BAP was needed to optimize embryo development • Addition of 1mg NAA on plantlet root formation led to fewer roots but longer root length
Sghaier et al. (2009)	Date palm 'Deglet Nour'	Friable callus	• Suspension culture: 2.25g MS salts, MS vitamins, Fe-EDTA 32 mg, 30g sucrose, 100mg *myo*-inositol, 2mg glycine, 100mg glutamine, 120mg KH2PO4, 30mg adenine, 1 mg 2,4-D, 300mg AC, light conditions ○ Comparison: 4, 30µM ABA, 3, 5mM arginine, 30, 60, 90g sucrose • Conversion media: SBM with 4.56mg MS salts, no PGR, and 8mg agar	• 20µM ABA increased embryo width and protein content • All treatments showed an increase in dry weight over untreated control, especially 90g sucrose and 20µM ABA treatments. Addition of either 4 or 20µM ABA increased proliferation rate • The storage protein glutelin was detected in 20µM ABA or 3mM arginine treatments
Zouine et al. (2005)	Date palm 'Jihel' 'Bousthami noir'	Shoot tips	• callus induction: MS, 30g sucrose, 0.15g AC, 6.8g carragennan, 14µM BAP, 22.5µM 2,4-D • SE induction: SBM with 0.28µM BAP and 2.25µM 2,4-D, dark conditions • Maturation: SBM with 1/2MS, 1.4µM BAP, 0.45µM 2,4-D, 0.25g AC, no gelling agents ○ Comparison: 1, 10, 100µM ABA on liquid or solid media ○ Comparison: 330, 670, 1340µM glutamine • Conversion: SBM with 1/2MS, no PGR ○ Comparison: 0.25 or 0.5g AC ○ Comparison: 6.8g carragennan or 2g phytagel	• Addition of ABA was beneficial for somatic embryo production over the same media without ABA • Somatic embryo development on solid media was slower than in liquid media • More somatic embryos were produced in liquid media • Histological studies showed that somatic embryos had fewer reserve compounds than zygotic embryos • Addition of either 0.25 or .5g AC and 2.5g phytagel may enhance conversion of somatic embryos
Abohatem et al. (2011)	Date palm 'Boufessous s' 'Bouskri'	Shoot tips	• Callogenesis induction: MS, 30g sucrose, 150mg AC, 7g agar, 5mg BAP, 5mg 2,4-D, dark conditions • SE induction: SBM with 0.1mg BAP and 0.5mg 2,4-D • Cell suspension: SBM without agar, 1/2MS, 0.1mg 2,4-D, light conditions ○ Comparison: 0.3, 0.4, 0.5mg BAP ○ Comparison: 7, 15, 20 day subculture intervals	• 0.2mg BAP produced the highest number of somatic embryos in comparison to the other treatments for both cultivars • Phenolic content and peroxidase activities increased with the longer 15 or 20 day subculture intervals, leading to tissue browning, whereas 7 day subcultures had reduced levels and an increased embryo proliferation
Al-Khayri (2011)	Date palm 'Nabout Saif'	Shoot tips	• Callus induction: MS salts, 170mg NaH2PO4, 125mg *myo*-inositol, 200mg glutamine, 5mg thiamine-HCl, 1mg nicotinic acid, 1mg pyridoxine-HCl, 30g sucrose, 1.5g AC 7g agar, 100mg 2,4-D, 3mg 2ip, dark conditions	• Addition of either additive, regardless of concentration, increased callus weight with increasing weight with increasing concentrations of either additive • Yeast extract improved embryo production with increasing concentration up to 1g, the highest tested

			• Callus proliferation: SBM with 10mg NAA and 30mg 2iP, light conditions
			• Embryogenic callus maintenance: SBM with 10mg NAA and 1.5mg 2iP
			  ○ Comparison: 0, 0.1, 0.25, 0.5, and 1g yeast extract
			  ○ Comparison: 0, 0.1, 0.25, 0.5, and 1g casein hydrolysate
				• Addition of casein hydrolysate improved embryo production, but not as much as yeast extract and little variation was seen between 0.1 and 1g

Khierallah and Hussein (2013)	Date palm 'Bream'	Shoot tips	• Callus initiation and proliferation: MS, 1mg thiamine-HCl, 1mg pyridoxine-HCl, 50mg adenine sulfate, 100mg *myo*-inositol, 170mg NaH2PO4 2H20, 200mg glutamine, 30g sucrose, 2.5g AC, 2g PVP, 7g agar, 50mg/L picloram, 3mg/L 2ip, light conditions • SE induction experimental media: SBM with 0, 5, 10, 20% coconut water or 0, 0.5, 1.0, or 2.0g/L casein hydrolysate • Conversion: SBM with 0.1mg/L NAA and 0.05mg/L BAP	• Both coconut water and casein hydrolysate treatments showed increased number of globular embryos and both fresh and dry weight of embryogenic calli • The highest concentration treatments for both coconut water and casein hydrolysate showed the best improvements compared to control

Othmani et al. (2009)	Date palm 'Boufeggous'	Juvenile leaves	• SE induction MS, 30g sucrose, 0.3g AC, 7g agar, dark conditions
			  ○ Comparison: 1, 5, 10, 50, and 100mg 2,4D, 1, 5, and 10mg picloram, a combination of 1mg 2,4D, 2mg NAA, and 1mg BAP, or no GR
			• Maintenance media: SBM with 0.1mg 2,4D
			  ○ Comparison: 6, 12, 24, and 48hr partial desiccation
			• SE maturation: SBM with 0.1mg AC, light conditions, with or without 1mg ABA depending on comparison treatment
			  ○ Comparison: effect of chopping with a scalpel blade or straining embryogenic calli through a fine mesh
			• Conversion: SBM with 1mg NAA and 0.1mg AC, light conditions
			• Acclimation: SBM with 1/2MS and 1mg IBA
				• Callus induction was only achieved with 1-50mg 2,4-D, picloram and lack of PGR did not produce embryogenic calli. 10mg 2,4D was the best treatment
				• Small leaf explants 5-10mm in length were more likely to form embryogenic tissue than larger explants
				• Fine chopping decreased the average time taken for embryo maturation and dramatically increased the average number of somatic embryos per 500mg fresh weight of embryogenic callus about eight times that of control
				• 6 to 12 hr. desiccation decreased the average time taken for embryo maturation and increased the average number of somatic embryos produced. 12 hour desiccation treatment was the best of the desiccation treatments. No embryos were produced from 48 hour desiccation treatment

Alkhateeb (2008)	Date palm 'Suckary'	Shoot tips	• Culture initiation: MS, 100mg 2,4-D, 3mg 2ip, dark conditions • Culture swelling media: SBM with 10mg NAA and 30mg 2ip, light conditions • Embryogenic callus formation media: SBM with 20mg NAA and 6mg 2ip, light conditions • Experimental media: ○ Comparison: Sucrose at 0, 30, and 60g sucrose or 2, 4, 6, 8, and 10% date palm syrup	• 60g sucrose had the highest number of somatic embryos • 30g sucrose had highest conversion rate, followed by 6% date syrup • 2% and 10% date palm syrup treatments had the lowest number of generated embryos • 4, 6, and 8% date syrup was comparable to 30 and 60g sucrose for number of regenerated embryos and dry weight • Authors conclude that date syrup at 6% can be used as a substitute for sucrose for date palm media

Guerra and Handro (1998)	Juçara	Mature zygotic embryo Immature zygotic embryo Inflorescences Seedling leaves	• Zygotic SE Induction: MS salts, MW vitamins, 30g sucrose, 1.5-3g AC, 50-100mg 2,4-D, picloram, or NAA, 6g agar • Inflorescence SE induction: SBM with 50mg 2,4D and 3mg 2ip • Leaf SE induction: SBM with either 10-20mg 2,4-D or NAA, liquid media with filter paper bridged • SE maturation: SBM with 2.5-5 mg 2ip and 0.1mg NAA • Conversion: ½ SBM free of PGR • Multiplication: SBM with 10mg 2,4-D and 3mg 2ip	• AC was essential for initiating cultures from zygotic embryo and inflorescence explants • High 2,4-D concentrations between 50 and 100mg were required to induce SE • Direct SE was observed • Plants were successfully regenerated from somatic embryos and acclimated to greenhouse conditions
Saldanha and Martins-Corder, (2012)	Juçara	Immature zygotic embryo	• SE induction: MS salts, MW vitamins, 30g sucrose, 0.5g glutamine, 1.5g AC, 7g agar, 3mg 2ip, 100mg 2,4-D, dark conditions o Comparison: additional 0, 2, 4 ,8, 12mM CaCl2.2H2O • Maturation: SBM with 3mg 2ip, 50mg 2,4-D • Conversion: SBM, light conditions o Comparison: MS and 1/2MS	• Calcium chloride did not affect SE induction, but did reduce the number of somatic embryos formed with fewer embryos produced as calcium chloride concentration increased • Embryo conversion was successfully achieved on either MS or 1/2MS without significant differences
Scherwinski-Pereira et al., (2012)	Açaí	Immature zygotic embryo	• SE induction: MS, 30g sucrose, 1.5g AC, 6g agar, dark conditions o Comparison: 0, 225, 450µM picloram or 2,4-D • SE Differentiation/Maturation: SBM with 0.537µM NAA and 12.3µM 2ip • Conversion: SBM with 1/2MS, 20g sucrose, and 2.5g AC, light conditions	• Overall, picloram was better than 2,4-D for producing primary callus. • Picloram was the only auxin to produce embryogenic callus. Best picloram concentration for embryogenic callus formation was 225µM • Plants were successfully regenerated from somatic embryos and acclimatized =
Ledo et al., (2002)	Açaí	Mature and immature zygotic embryos	• SE induction: MS, 30g sucrose, 0.5g hydrolyzed casein 2.5g AC, 6g agar, dark conditions 7 days, light thereafter o Comparison: 113, 226, 339, 454, 565, 678µM 2,4-D • Maturation: SBM with 0.537µM NAA and 12.3 µM 2ip • Conversion: SBM with 1/2MS and 15g sucrose	• For mature zygotic embryos, 2,4-D treatment concentrations between 339µM and 565µM led to formation of granular structures and globular somatic embryos • Highest embryogenic efficiency for mature zygotic embryo explants occurred with 339µM 2,4-D • Despite forming granular structures, no explants formed globular somatic embryos
Sarasan et al. (2002)	Bottle palm	Zygotic embryos Undivided seedlings Divided seedlings	• SE induction: MS o Comparison: 30g sucrose or maltose o Comparison: Solid or liquid media o Comparison: with or without 2g AC o Comparison: 0.1, 0.5, 1, 2, 3, 5mg 2,4-D • Conversion: MS, 30g sucrose	• AC led to callusing, browning, and loss of embryogenic potential • Sucrose superior to maltose • More somatic embryos formed on undivided seedlings than on divided seedlings • Liquid media increased culture browning

			○ Comparison: 0.1, 0.5, 1 and 3mg BAP or 0.5mg BA with either 0.1, 0.5, 1 and 3mg NAA or 0.1, 0.5 and 1mg GA3	• Low concentrations of 2,4-D between 0.1 and 2mg resulted In fewer embryos and higher concentration (5mg) resulted in callusing and rapid tissue browning • Addition of cytokinins led to rapid tissue browning • 7% of embryos on conversion media with 0.5mg BA developed into plumular embryos, addition of GA3 reduced browning but increased callusing • Plants were not successfully regenerated from somatic embryos
Moura et al. (2009)	Macaw palm	Mature zygotic embryos	• Callus induction: Y3 salts, 68.46g sucrose, 1g casein hydrolysate, 100g *myo*-inositol, 2.5g gelrite, dark conditions ○ Comparison: 9 µM of 2,4-D, picloram, NOA, or CPA with or without 1 µM TDZ • Callus multiplication: SBM with the same 2,4-D and Picloram treatments ○ All treatments with or without 3g AC • Somatic embryo induction: SBM, all treatments with AC transferred to light • Secondary SE: SBM with 9 or 20 µM picloram and 300mg AC, dark conditions • Conversion: SBM with 3g AC, no GR	• Within treatments with TDZ, the most effective auxin was 2,4-D, without TDZ, the most effective was picloram • Neither NOA or CPA were as effective as 2,4-D or picloram, regardless of TDZ • Including AC improved callus multiplication • 2,4-D was more effective than picloram for multiplying callus cultures • TDZ was detrimental for callus for multiplication • Picloram treatments were the only treatments to have successful SE induction • Half of the somatic embryos placed on conversion media began to convert, but few fully completed the process
Padilha (2013)	Macaw palm	Germinated seedling TCL	• Callus Induction: Y3 salts, MW vitamins, 30g sucrose, 1.5g AC, 0.5g glutamine, 2g Gelzan ○ Comparison: 150, 300, 600µM picloram ○ Comparison: MS or Y3 with 150 or 300µM picloram ○ Comparison: with or without 1µM silver nitrate • Multiplication/Maturation: SBM with 1g glutamine and 500mg casein hydrolysate, 0.5µM NAA ○ Comparison: 0, 6.25, 12.5, 25µM 2ip ○ Comparison: 2ip or BAP at 12.5 or 25µM, all treatments with 75µM picloram • Conversion: SBM with 500mg casein hydrolysate, 0.5µM NAA, and 25µM 2ip, light conditions	• Any of the tested concentrations of picloram promoted primary calli induction, although 150 µM promoted calli growth in a wider range of TCL cuts further from the meristem region • Y3 at each compared picloram concentration had increased percentages of explants that formed callus compared to MS • Y3 cultures were the only cultures to form somatic embryos • Silver nitrate had a negative effect on both callogenesis and SE • 12.5µM of either 2ip or BAP had the highest rate of somatic embryo formation
Balzon et al. (2013)	Oil palm	Mature zygotic embryos	• Calli induction: MS, 2.5g phytagel, 30g sucrose, dark conditions ○ Comparison: 450µM 2,4-D or picloram ○ With or without 2.5g AC • Callus proliferation: SBM with 10µM 2ip	• Callus induction was improved with addition of AC, no embryogenic call growth was seen on 2,4-D treatment without AC • Picloram was more effective than 2,4-D for callus induction

			○ Comparison: 40µM 2,4-D or picloram • SE differentiation: SBM with 12.3µM and 0.54µM • Conversion: SBM with 1/2MS salts, no GR, 20g sucrose, and with 2.5g AC, light conditions	• No significant difference was calculated between picloram or 2,4-D for number of formed somatic embryos, although regeneration frequency, in terms of number of total regenerated plants divided by total number of somatic embryo, was higher in picloram treatments
Jaynathi et al., (2011)	Oil palm Two hybrids of 'Dura X Pisifera'	Immature zygotic embryo cotyledonary nodes	• SE induction: Y3, 30g sucrose, 0.5g casein hydrolysate, 20g AC, 8g agar, 4µM 2,4-D, 40µM NAA, 10µM 2,4,5-T, 10µM TDZ, 10µM BAP, dark conditions • Maturation: SBM with 10µM BAP and 40µM ABA, light conditions • Conversion: SBM with 2µM BAP and 1µM ABA, light conditions	• Direct SE observed • Secondary SE occurred in 2% of somatic embryos • 80% of explants for one hybrid and 100% of the other produced embryogenic tissue • Both hybrids produced somatic embryos, which were successfully regenerated into whole plants
Rajesh et al. (2003)	Oil palm	Mature zygotic embryos	• Callus induction: MS, 30g sucrose, 5.5g agar, 113.12µM 2,4-D, 4.76µM 2ip, dark conditions • Regeneration: SBM with Blaydes medium and 0.045µM 2,4-D, light conditions ○ Comparison: 4.54µM TDZ, 2.85µM zeatin riboside, 1mM putrescine, 100µM spermine • Rooting: liquid Blaydes medium with 4.9mM IBA	• 1mM putrescine followed by 100µM spermine and 2.85 µM zeatin riboside treatments had the highest average numbers of embryogenic calluses • 1 mM putrescine had the highest average number of somatic embryos at 5.6 with 100µM spermine with the second highest at 2.13 • Secondary SE only observed in polyamine treatments • Plants were successfully regenerated and acclimatized to environmental conditions
Teixeira et al. (1993)	Oil palm 'Tenera' 'Dura' 'Pisifera'	Immature and mature zygotic embryos	• Induction: Y3, 30g sucrose, 500mg cysteine, 5g PVP, 3g AC, 2.2g gelrite, 500µM 2,4-D, dark conditions • Maturation: SBM with 15µM NAA and 2µM ABA • Conversion: SBM with MS and no GR	• Embryos older than 140 days post- pollination did not form embryogenic tissue • Embryos collected between 77 and 128 days post-pollination grew embryogenic tissue with the highest percent at 91 days and 100 days post-pollination • Friable embryogenic tissue gave rise to somatic embryos which were successfully regenerated into whole plantlets
Thuzar et al. (2011)	Oil palm 'Tenera'	Zygotic embryos	• Callus induction: 30g sucrose, light conditions ○ Comparison: SBM with MS or N6 media, each with 2mg of picloram, 2,4-D, or dicamba • SE induction: SBM with N6 salts, 0.1mg 2,4-D, 0.16g putrescine, 0.5g casein hydrolysate, and 2g AC • Regeneration media: SBM with 0.5g AC ○ Comparison: N6, modified N6, 1/2MS, full MS, 1/2MS with 0.5g NAA and 1mg BAP	• N6 salts were more effective than MS for callus induction • 2,4-D was the most effective auxin for embryogenic callus induction, followed by dicamba and then picloram • The modified N6 (see reference for composition) showed the highest root and shoot formation with the highest regeneration rate • Plants were successfully regenerated and acclimated to greenhouse conditions

Steinmacher et al. (2007)	Peach palm	TCL of shoot meristem region from germinated seedlings	• Zygotic embryo germination: MS, MW vitamins, 30g sucrose, 1.5g AC, 7g agar, light conditions • Calli induction: SBM without agar and with 0.5g glutamine and 2.5g phytagel, dark conditions ○ Comparison: 0, 150, 300, or 600µM picloram • Maturation: SBM with 40µM 2,4-D, 10µM 2ip, 1g glutamine, 0.5g casein hydrolysate, dark conditions • Conversion: SBM with 24.6µM 2ip and 0.44µM NAA	• Picloram was essential for culture initiation, with all picloram treatments seeing tissue response • TCL regions with the highest formation of primary calli were sections from the meristem and then sections directly above and below with quickly decreasing response rates further from the meristem • Meristematic regions gave rise to the highest percent of explants forming embryogenic calli with the 300µM picloram treatment having the highest • Plants were successfully regenerated from somatic embryos and acclimatized
Steinmacher et al. (2011)	Peach palm	Mature zygotic embryo	• SE induction: MS salts, MW vitamins, 30g sucrose, 500mg glutamine, 2.5g gelrite, 1µM silver nitrate, and 10µM picloram, dark conditions • Multiplication: SBM ○ Comparison: Solid or liquid media in a twin flask temporary immersion system (TIS) • Maturation: SBM with 40µM 2,4-D and 10µM 2ip, 1.5g AC, 1g glutamine, 500mg hydrolyzed casein • Conversion: SBM with 20µM 2ip and 0.5µM NAA, light conditions • Secondary conversion: SBM with 1.5g AC	• Secondary SE observed • TIS greatly improved the number of somatic embryos produced during multiplication • TIS increased plantlet growth in both height, rooting %, and # of roots per plantlet • Survival rate of plantlets from somatic embryos cultured on solid media was significantly higher than plantlets from TIS system
Valverde et al. (1987)	Peach palm	Shoot tips	• Induction: Liquid modified MS salts, 100mg *myo*-inositol, 0.4mg thiamine-HCl, 0.5mg pyridoxine-HCl, 0.5mg nicotinic acid, 30g sucrose, 15% coconut water, 0.06mg picloram, 5mg BAP, dark conditions then transfer to light conditions • Secondary media: SBM with 500mg casein hydrolysate, 1g glutamine, 120mg arginine, and 2mg glycine • Rooting media: SBM without GR and with 0.5mg AC	• Both SE and organogenesis were observed, including both from the same callus • Organogenesis was more common than SE • Plantlets were regenerated and acclimatized to greenhouse environment

Goh et al. (2001)	*Calamus merrillii* and *C. subinermis*	Root tip fragments
		Young unopened leaves
		Zygotic embryos
			• Induction: MS, 100mg *myo*-inositol, 500mg casein hydrolysate, 2mg glycine, 1mg thiamine, 1mg pyridoxine-HCl, 1mg nicotinic acid, 30g sucrose, 7g agar, dark conditions
			  ○ Comparison: 2.5, 5, 5.7mg picloram
			• Maturation: SBM
			  ○ Comparison: 1, 2.5, 5mg picloram
			• Conversion: SBM, light conditions
				• Zygotic embryo and root tip explants of both species grew calli, although only *C. merrillii* leaf explants responded
				• Reducing picloram concentrations stimulated maturation of bipolar embryos
				• Addition of GA and AC to media stimulated root growth from somatic embryos
				• Only a few *C. subinermis* plantlets from somatic embryos reached development stage for acclimatization

				• Histological analysis showed an apparent lack of starch and protein reserves • Some somatic embryos displayed multiple shoot apices
Alang and Krishnapillay, (1987)	Sago palm	Stem apex and unemerged leaves	• Induction: MS. 7g agar ○ Comparison: with 3g AC 50-200mg/L, IAA, NAA, IBA, or 2,4-D, without AC, 5-25mg/L ○ Comparison: 1-3mg Kinetin, 2ip, or BAP ○ Comparison: light or dark • Conversion: SBM with 50mg 2,4-D, 3mg 2ip	• High 2,4-D and AC were essential for culture initiation • Friable tissue was easily separated and easy to multiply • Nodular structures developed into plantlets after transfer to conversion media
Gallo-Meagher and Green (2002)	Saw palmetto	Immature zygotic embryo	• Induction: MS, 30g sucrose, 1.5g AC, 2.5g phytagel, 452µM 2,4-D, 14.7µM 2ip, dark conditions • Multiplication: SBM with 90.4µM 2,4-D • Conversion: SBM, light conditions ○ Comparison: 0, 0.9 or 9µM TDZ ○ Rooting: SBM with 22.2µM NAA	• Direct SE observed • 12% of somatic embryos on media with 0.9µM TDZ produced complete plantlets while somatic embryos 9µM TDZ did not regenerate • Highest shoot production, 35%, was seen on somatic embryos cultured on conversion media without TDZ • 100% rooting achieved when plantlets were transferred to media with 22.2µM NAA

1 1- Media contains this specified basal salt and vitamin mix unless otherwise stated. 2-All values are in terms of amount per liter of media. 3- Same basic media (SBM) signifies that the same media (salt mixture, vitamins, g/L sucrose, etc) is also being used in this step. The first media in each reference, usually an induction step, is the same basic media for each subsequent step within this reference unless specified. 4-Plant growth regulator refers to auxins, cytokinins, gibberellic acid, or ABA

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2. Protoplast isolation and culture using embryogenic cultures of three native Brazilian palm species: Peach palm (*Bactris gasipaes*), Butiá-da-serra (*Butia eriospatha*), and açaí *Euterpe oleracea*)

Abstract

In addition to being a source of tissue regeneration, protoplasts could be used to create hybrids with novel traits through somatic fusion. However, factors affecting protoplast isolation and culture have only been studied in several key economic palm species. Three native Brazilian palm species, peach palm (*Bactris gasipaes*), butiá-da-serra (*Bactris eriospatha*), and açaí (*Euterpe oleracea*), were placed in enzymatic incubation for protoplast isolation. Cultures were left to incubate either on an orbital shaker or left stationary for several time increments. It was found that stationary incubation produced few protoplasts compared to orbital shaking. The highest protoplast yields were $5.50\pm0.68x10^5$ cells/gram FW for peach palm at six hour, $5.36\pm2.23x10^5$ cells/ gram FW for butiá at 24 hour, and $1.22\pm0.13x10^6$ cells/gram FW for açaí at six hour incubation with orbital shaking at 45 rpm. However, drastic drops in protoplast yield were observed for both açaí and peach palm after six hour orbiting incubation. Cell viability was high for all three species in both stationary and orbiting incubation, but açaí viability began to diminish rapidly after six hours of orbiting incubation. Viability remained high for all three species during stationary incubation. Both 1µM and 10µM picloram were found to promote early resumption of cell division in all three species and nearly essential for second and third cell divisions, however 2µM 2-ip was not found to influence cell activity. Further optimization will be required to regenerate microcalli, which can be cultured to regenerate somatic embryos and eventually give rise to fertile plants.

Introduction

Uses for protoplasts

Protoplasts, cells enzymatically removed from tissue, provide several key technologies that may prove useful to humans. This is especially true for palm species, which are often propagated principally through tissue culture via somatic embryogenesis (SE). One application is somatic hybridization, the combination of some or all parts of two protoplasts from separate, but related, species, to create hybrid plants that circumvents barriers to cross-pollination, such as incompatibility and

non-overlapping pollination periods (Grosser and Chandler, 1987). For example, somatic hybrids of *Solanum melongena* and *S. sisymbrifolium* were created for resistance to bacterial and fungal wilts (Collonnier et al., 2001) and hybridization between citrus cultivars is of particular commercial interest for increased disease resistance and new cultivars (see de Bona et al., (2011) for review). The same might be applied to palms, which of whom have long life cycles that make conventional breeding difficult.

A second method is to regenerate somatic embryos from protoplasts. While SE can often be reliably induced using either zygotic embryos, as in peach palm (Steinmacher et al., 2007a), or shoot apical meristems, such in date palm (*Phoenix dactylifera*) (Bhaskaran and Smith, 1992), each tissue has distinct disadvantages; zygotic embryo response depends heavily on maturity (Teixeira et al., 1993) and are disadvantageous for breeders due to being genetically different from their parents and many palms have only one shoot apical meristem, whose harvest kills the plant. Protoplasts do not have these inherent disadvantages, though their culture is often dependent on many factors, such as tissue of origin, growth regulators, genotype effect, culture methods, and cell density, many of these such factors can be optimized *in vitro*. Protoplasts have been isolated and cell division induced from several palm species, such as oil palm (*Elaeis guineensis*) (Sambanthamurthi et al., 1996), date palm (Chabane et al., 2007), and guadalupe palm (*Erythea edulis*) (Gabr and Tisserat, 1984). To date there is only one published account of full plant regeneration from protoplasts isolated from suspension cultures of oil palm (Masani et al., 2013), however SE has been successfully induced in other species, including tobacco (*Nicotiana tabacum*) (Berry et al., 1983), multiple *Daucus* specie (Maćkowska et al., 2014), white spruce (*Picea glauca*) (Attree et al., 1987), and barley (*Hordeum vulgare*) (Kihara and Funatsuki, 1995). A third possible application of protoplast culture, as suggested by (Aoyagi (2011), is encapsulation of plant protoplasts in an alginate matrix with cell wall inhibitors as a means of generating useful metabolites for pharmaceuticals, rather than from wild sources.

Factors involved in protoplast isolation and culture

Protoplast isolation cell yield is heavily influenced by enzyme solution composition. Type and concentration of enzymes, osmotic pressure, presence of antioxidants, salts concentration, incubation time, and stationary vs. moving incubation can affect the total yield of protoplasts, as well as their viability(for review, see (Davey et al., 2005). Enzymes, such as cellulase, pectinase, and hemicellulase are required to break down components of the cell wall (Assani et al., 2002), to release the protoplast into the surrounding solution. High osmotic pressure in the form of high concentrations of mannitol or sucrose is required to maintain cell integrity after the cell wall is removed (Jianbo et al., 2011). During incubation, the isolated protoplasts are vulnerable to cell lysis due to lack of protective cell walls. Insufficient incubation time can lead to low cell yields, but longer incubation times tend to also decrease cell yield (Chabane et al., 2007). After filtration, protoplasts can be cultivated in several ways, such as suspended in liquid culture or embedded in alginate or agarose. However, just as auxins are often required to start culture initiation, such as in macaw palm (*Acrocomia aculeate*) (Moura et al., 2009), it was found that auxins were necessary for sustained cell division in mulberry (*Morus indica*) (Umate et al., 2005).

Applications of protoplast culture to three native species

The goal of this work was to investigate protoplast isolation for regeneration of somatic embryos in three native Brazilian palms of some level of commercial interest: peach palm (*Bactris gasipaes*), butiá-da-serra (*Butia eriospatha)* and açaí (*Euterpe oleracea*). Three species, each with previously established cultures disiplaying different morphologicl characteristics and embryogenic potential, were selected in order to find trends in protoplast isolation and culture that can be used in multiple species. Peach palm, a native Amazonian palm used as source of food, fibers, and a potentially sustainable source of heart-of-palm, has a well-developed SE induction protocol (Heringer et al., 2014; Steinmacher et al., 2007b, 2011). Another native palm, açaí, is cultivated for its fruit and heart-of-palm and has had successful SE protocols developed using zygotic embryos (Ledo et al., 2002). Butiá-da-serra, referred hereafter as simply butiá, is a threatened native of the Mata Atlantica in the southern

region of Brazil known for its sweet fruit, although it is not widely cultivated. No work has been published on protoplast isolation from any of these three species. In order to contribute both to more understanding on methods of successful isolation and cultivation of palm species, several factors were tested in all three species. Of particular interest is possibly creating somatic embryos in recalcitrant fast-growing butiá cultures through immobilization of single cells in the hope that the lack of competition would lead to embryo differentiation.

Methods

Plant material

Peach palm cultures were previously established from excised zygotic embryos from fruits collected about 12 weeks after pollination from the peach palm germplasm bank of the Nacional de Pesquisas da Amazonia (INPA), Manaus, Brazil and cultured as described in Heringer et al., (2014). Butia-da-serra and acai apical meristems were harvested from one to two year old immature palms and cultured according to the thin cell layer method described in Steinmacher et al., (2007c). Embryogenic 'Jirau 3' peach palm and fast-growing butiá callus cultures were multiplied on media consisting of Murishige and Skoog (MS) (Murashige and Skoog, 1962) media with Morel and Wetmore (WM) (Morel and Wetmore, 1951) vitamins, 30g/L sucrose, 1g/L glutamine, and 10µM picloram. Previously established açaí cultures were multiplied on the same media except for an additional 2.5g/L activated charcoal and increased picloram to 50µM. One gram of tissue was collected and placed in a 50mL centrifuge tube containing 10mL of plasmolysis solution consisting of MS salts without calcium and half ammonium nitrate, MW vitamins, 0.2M glucose, 0.3M mannitol, 1g/L glutamine, 1g/L myo-inositol, and 200mg/L ascorbic acid for one hour.

Protoplast isolation

Afterwards, the plasmolysis solution was pipetted off and replaced with filter sterilized enzyme solution similar to plasmolysis solution, except for addition of 2% w/v cellulase, 0.5% hemicellulase, and 0.5% pectinase and then either incubated stationary for 6, 12, 18, or 24 hours or on an

orbital shaker at 45 rpm for 3, 6, 12, or 24 hours in the dark at 25±2 °C. The resulting protoplast solution was then poured through a 40µm nylon filter into a sterile 50mL centrifuge tube and then centrifuged at 100*xg* for eight minutes. The supernatant was removed and the pellet washed with 10mL of plasmolysis solution and the pellet gently resuspeded. After gentle mixing, the solution was centrifuged again at 100xg for eight minutes and the supernatant was pipetted away. Two mL of resuspension solution consisting of plasmolysis solution with an additional 0.5g/L malt extract and 0.5g/L yeast extract was used to resuspend the protoplast pellet. Fifty microliter aliquots of each individual preparation were removed for cell yield estimation in hemocytometer using an Olympus IX81 inverted microscope (Shinjuku, Tokyo, Japan). Viability was checked using 0.1mg/L fluorescein diacetate (FDA) (Figure 2-1). Four hemocytometer grids were used per separate protoplast isolation to estimate cell yield.

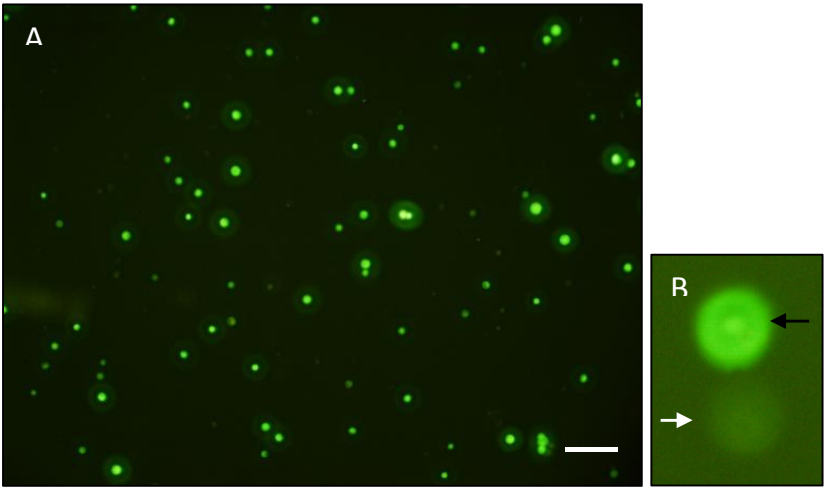

Figure 2-1: (A) Viable fluorescing spherical butiá protoplasts (bar = 200um). (B) A viable butiá protoplast (Black arrow) and a non-viable non-fluorescing butiá protoplast (White arrow).

Protoplast culture

Platting density was adjusted to 2 x 10^5 cells/mL before mixing with equal volumes of 4% alginate dissolved in plasmolysis solution. Alternatively, the same density of protoplasts was mixed with warm 1.2% agarose dissolved in plasmolysis solution. The protoplast-alginate solution were added drop-wise to a 50mM $CaCl_2$ solution with modified MS salts, 0.2M glucose, and 0.3M mannitol and the resulting beads were allowed to polymerize for fifteen minutes. The beads were then removed from the solution and then placed in 10mL of culture media consisting of MS salts, MW vitamins, 1g/L glutamine, 0.5M glucose, 1g/L glutamine, and either 0, 1, or 10µM picloram with or without 2µM 2-ip in test tubes (5 beads/tube) and left stationary in a dark growth chamber at 25 ±2 °C. Additionally, two beads per treatment were collected and placed in twelve-well assay plates to observe days until first signs of cell division. Agarose-protoplast beads were cultured exclusively in twelve-well assay plates with the same liquid media.

Data analysis

Three replicate protoplast isolations were performed for each incubation time for both orbital and stationary incubation. Protoplast yield was calculated as described in Masani et al., (2013). Viability was calculated by dividing the number of fluorescing cells by the total number of counted cells. Cell division was calculated by observing alginate beads under an inverted microscope for the first, second, and third cell divisions for the first week. Each growth regulator treatment consisted of three replicates of two individual alginate beads each. Cell division was monitored daily for the first week using an inverted microscope. Alginate beads showing at least three first, second, and third cell divisions were recorded and the average number of days for each cell division calculated after a week. Resulting data was analyzed using R software to compare number of days required for first, second, and third divisions by growth regulator concentratiosn for each individual species. Statistically significant ($p<0.05$) results were further analyzed using the Tukey-Kramer post hoc test. The percent of alginate beads showing each type of cell division was also recorded.

Results and Discussion

Cell yield

Stationary incubation yielded far fewer cells than incubations placed on a rotary shaker regardless of incubation time or species (See Figure 2-2 and 2-3). Six hour stationary incubation produced the fewest protoplasts for all three species. No butiá protoplasts were detected in any of the repetitions at this incubation time. As stationary incubation time increased, protoplast yield for each species tended to increase as well. The highest stationary incubation yields for peach palm, butiá, and açaí were at 24 hours incubation with a total of $2.64\pm0.62x10^4$, $5.83\pm0.42x10^4$, and $2.00\pm0.29x10^4$ cells/gram FW, respectively. However, orbital shaking produced higher total numbers of protoplasts even after three hours of incubation with $1.75\pm0.29x10^5$, $1.13\pm0.27x10^5$, and $1.94\pm0.37x10^5$ protoplasts/gram FW for peach palm, butiá, and açaí, respectively. Unlike stationary incubation, total yield did not always increase with increasing shaking incubation times. For both peach palm and açaí, optimum incubation time was six hours with a total highest yield for each of the two species of all treatments at $5.50\pm0.68x10^5$ and $1.22\pm0.13x10^6$ protoplasts/ gram FW, respectively. These data vary somewhat with results in other species. Date palm protoplasts in stationary incubation had a yield of $5.6x10^5$ cells per g FW in 'Deglet nuor' and $4.95x10^5$ cells per g FW in 'Takerboucht' nodular callus after 12 hours of incubation (Chabane et al., 2007). In the same report, the effect of incubation time on yield found that the highest yield was at the lowest time interval, 12 hours, and then decreased to barely detectable between 16-20 hour incubation. (Gabr and Tisserat, 1984) found that protoplast yield from Guadalupe palm (*Erythe edulis*) for 24 hours was highest when placed in stationary incubation and yield decreased with increasing agitation between 50-150rpm. Fourteen hours of stationary incubation was sufficient to obtain $1.14x10^6$ cells/gram FW from oil palm suspension cultures (Masani et al., 2013), which, until this report, was the highest protoplast yield in palms. However, these yields are not as high as achieved in other species, as demonstrated by the isolation of $2.5x10^7$ cells/gram FW 'Hamlin' sweet orange (*Citrus senensis*) protoplasts from suspension cells after 16 to 18 hours at 45rpm (Niedz, 1993). Agitation for several hours has yielded large amounts of protoplasts in other species.

Four to five hours of incubation at 90 rpm agitation were sufficient to generate $1x10^6$ cells/g FW from garlic (*Allium sativum*) tissue (Hasegawa et al., 2002). Low incubation time of only 90-120 minutes on an orbital shaker at 100 rpm, however, only yielded $1x10^4$ cells/gram FW from oil palm suspension cultures, although the enzyme mix used only contained 10g/L driselase with no addition enzymes, including the almost universally added cellulase (Bass and Hughes, 1984). Following six hours of incubation time, peach palm and açaí incubations has dramatic drops in total protoplast yield. However, Increasing levels of cellular debris were observed during cell yield counting as incubation time increased. It is possible that the debris might act as a mechanical shearing force, which could possibly damage protoplast during shaking, leading to cell membrane lysis and death. Unlike these two species, butiá yields did increase with time with the highest protoplast yield at $5.36\pm2.23x10^5$ cells/gram FW after 24 hours of orbital shaking.

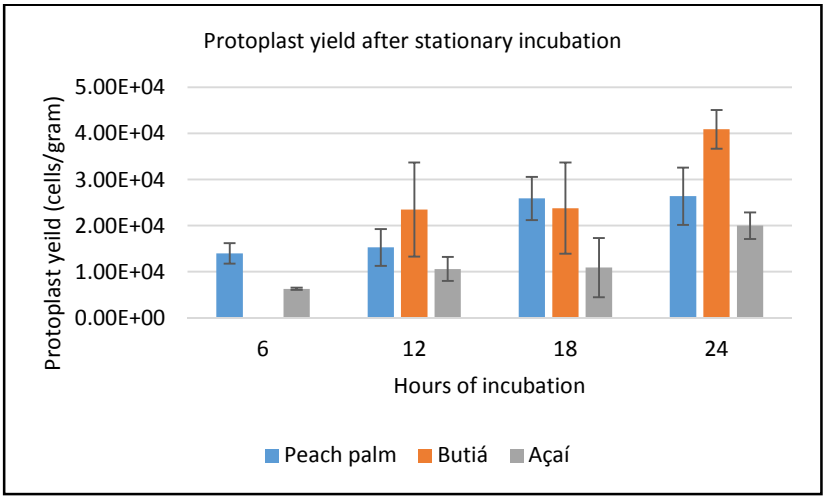

Figure 2-2: Protoplast yield by palm species and incubation time after stationary incubation in enzyme solution. Cell counts were estimated using counted number of cells in a hemocytometer.

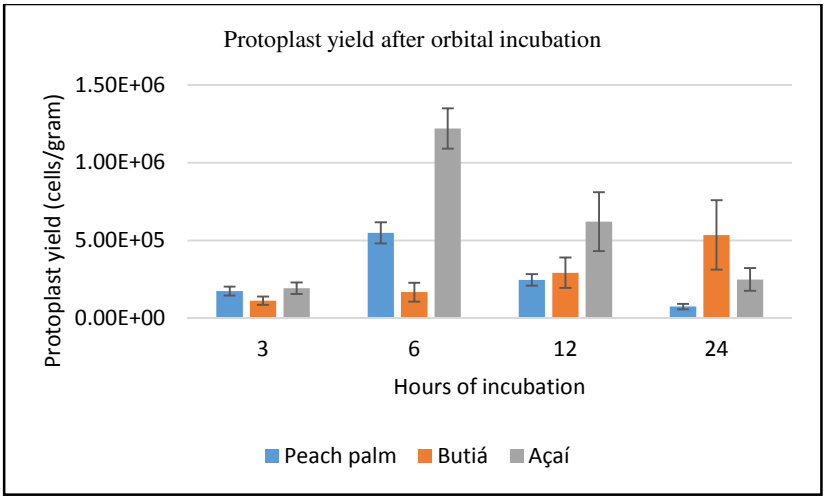

Figure 2-3: Protoplast yield by palm species and incubation time after orbital incubation at 45 rpm in enzyme solution. Cell counts were estimated using counted number of cells in a hemocytometer.

Butiá cell yields were low in comparison to either açaí or peach palm for both incubation methods. This was unexpected because the friable callus ideal for creating suspension cultures has been considered an excellent tissue for protoplast isolation (Folling et al., 1995). However, the friable butiá callus has been frequently observed to have a thick extracellular matrix, described in maize callus as a "net" of microfilaments, lipophilic substances, arabinogalactan proteins, and other substances (Samaj et al., 2008). Extracellular matrices are a common characteristic of many palm cultures, including *Calamus merillii* (Goh et al., 2001), *Acrocomia aculeate* (Moura et al., 2010), peach palm (Steinmacher et al., 2012), and occasionally observed on açaí embryos (personal observation, data not shown). This layer might act as a barrier that blocks enzymes from degrading cell walls. However, as shown as the gradual cell yield increase, this layer may act only to delay, but not wholly prevent, cell wall digestion.

Viability

Viability remained relatively high for all stationary incubation times with only slight fluctuation (See Figure 2-4 and 2-5). Peach palm protoplast viability during stationary incubation remained within 95-85% viability range, although a slight decrease was observed at 24 hour incubation. Butiá cells were not recovered at 6 hours of stationary incubation, however viability ranged from ~80-90% for 12, 18, and 24 hour incubation times. Açaí protoplast maintained 75-85% viability during all stationary incubation times. Likewise, viability remained high for peach palm and butiá under agitation incubation, however, açaí protoplast viability dramatically began to decline after 6 hours of incubation from ~90% viability for 3 and 6 hours to 68.3±9.18% viability at 12 hours and, finally, 34.0±2.52% at 24 hour incubation. Rather than the possible effect of cell debris in lysing protoplast, tissue oxidation might have led to decrease in viability. During filtration, the recovered açaí cultures appeared to be increasingly darker than in lower incubation times, which could show that tissue was oxidizing. This might indicate that although protoplasts were being liberated, despite high amounts of cellular debris,

that the isolated protoplasts were suffering the effects of tissue oxidation and had become inviable.

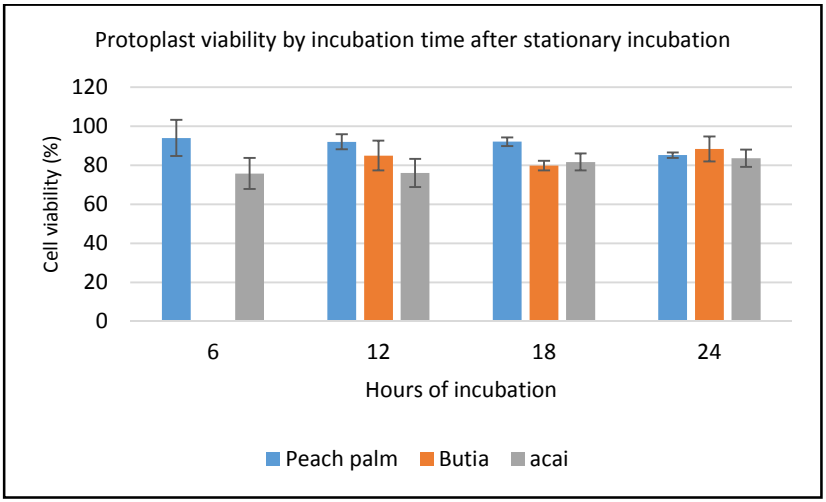

Figure 2-4:Protoplast viability after stationary incubation in enzyme solution for 6, 12, 18 or 24 hours. Viability reflects the proportion of cells fluorescing with FDA against total number of cells. No butia protoplasts were retrieved for 6 hour incubation

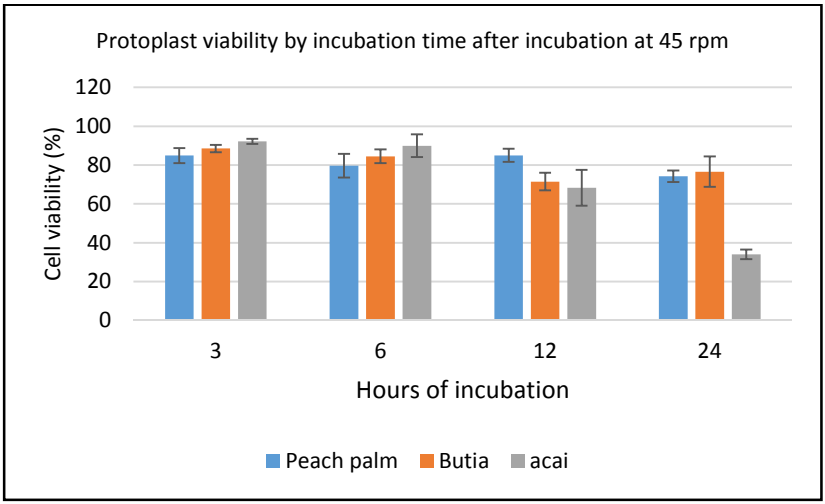

Figure 2-5: Protoplast viability after orbital incubation at 45rpm in enzyme solution for 3, 6, 12, or 24 hours. Viability reflects the proportion of cells fluorescing with FDA against total number of cells.

Effect of growth regualtors on protoplast cell division

Cell division was observed earlier in alginate beads with increasing levels of picloram with or without 2-ip (See table 2-1).

First signs of cellular division in butiá protoplasts embedded in alginate beads were observed in media containing 0, 1, and 10µM picloram 5.44, 4.08, and 3.58 days post isolation on average without 2µM 2ip and 5.22, 4.18, and 3.67 days with 2µM 2-ip. This trend was repeated in both peach palm and açaí. Peach palm protoplasts began division starting from 5.38, 4.72, and 4 days without 2µM 2-ip and 5.29, 4.0, and 4.0 days in media with 0, 1, and 10µM picloram, respectively. Açaí protoplasts began cell division starting from 5, 5, and 4.67 days without 2-ip and 5.5, 5, and 4.67 days with 2-ip in media with 0, 1, and 10µM picloram, respectively. As picloram concentration increased, there was a general trend of decreasing number of days until the first cell divisions were noticed in all three species, with or without 2-ip. 2-ip had no significant effect in stimulating cells to reenter the cell cycle. Picloram was found to have a significant effect on decreasing the amount of time until first cell divisions occured

in both peach palm ($p<0.001$) and butia ($p=0.002$), but not in açaí protoplasts ($p=0.693$). Subsequent Tukey-Kramer post hoc tests found that 1µM or 10µM were significantly different compared to media without picloram for both butia and peach palm. In all comparisons, 2ip was found not to have a significant effect.

Table 2-1: Average number of days until first, second, and third cell divisions were observed in alginate beads showing cell division

Species	Cell Division	Average number of days until type of cell division observed (µM 2-ip – µM picloram)					
		0-0	0-1	0-10	2-0	2-1	2-10
Butiá	First	5.44 A	4.08 B	3.58 B	5.22 A	4.18 B	3.67 B
	Second	6 A	5.86 A	4.83 B	6 A	5.67 A	5.25 B
	Third	None	6.66	6	None	6.67	6.33
Peach Palm	First	5.38 A	4.72 B	4 B	5.29 A	4 B	4 B
	Second	7	5.5	5.08	6	5.2	4.64
	Third	None	7	6	None	6	5.4
Açaí	First	5	5	4.67	5.5	5	4.67
	Second	None	6	5.33	6	5.67	5.38
	Third	None	6	6.33	None	6	6

Note: Letters represent statistically significant differences (alpha=0.05) determined through Tukey-Kramer post hoc test for each species and each type of cell division. Lack of letters indicates that no significant differences were found.

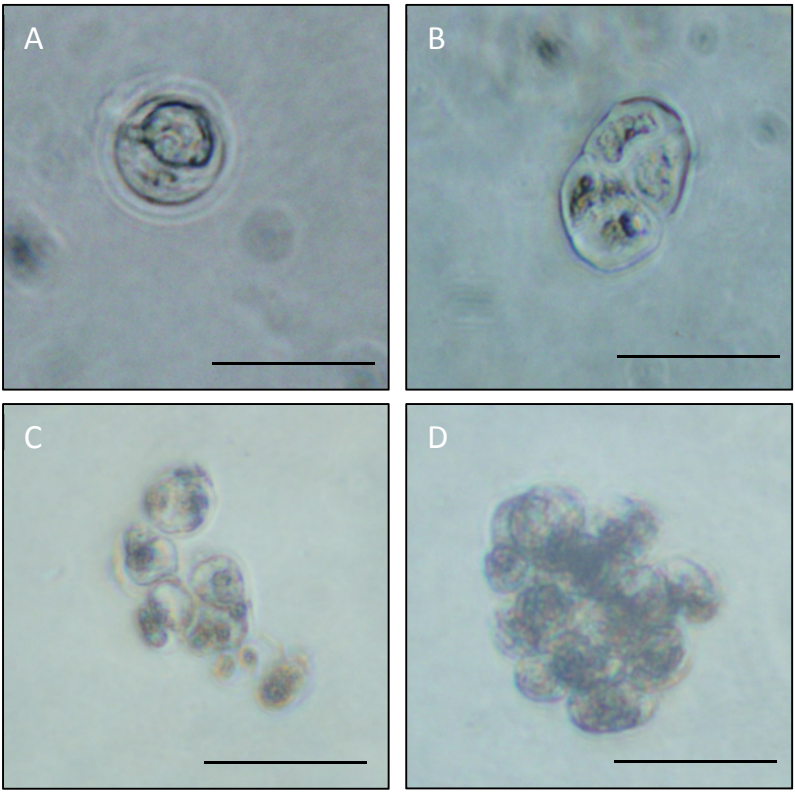

Figure 2-6: Stages of peach palm protoplast division and growth. (A) A single protoplast displaying a distinct nucleus and several organelles (One day post isolation) (bar= 10µm); (B) A cluster of four cells (Eight days post isolation) (bar= 20µm); (C) A small microcolony composed of several cells of different sizes (twelve days post isolation) (bar= 20µm); (D) Larger microcolony composed of many densely-packed cells (Twenty two days post isolation)(bar= 20µm)

Second and third divisions were observed within several days of the first signs of cell divisions in all species. However, no third cell divisions were observed in media without picloram. Auxins, such as 2,4-D and piclroam, have been frequently been shown to be essential for SE, as shown in sago palm (Metroxylon sagu) (Alang and Krishnapillay, 1987), açaí (Ledo et al., 2002), and oil palm (da Silva Guedes et al., 2011), and this likely is

an important additive for protoplast culture, as supported by Umate et al (2005), who observed that 13.5µM dicamba was essential for sustained cell division in mulberry (*Morus indica*).

Most alginate beads embedded with butiá protoplasts showed cell divisions (Figure 2-8, 2-9, 2-10), with 100% of all beads showing at least three cell divisions within the first week in 0µM 2-ip-1µM picloram, 0µM 2-ip-10µM picloram, and 2µM 2-ip-10µM picloram and 91.7% of 2µM 2-ip -1µM picloram beads showing cell division. However, only 75% of beads of 0µM 2-ip-0µM picloram and 2µM 2-ip-0µM picloram beads showed any sign of cell division in the first week. Second and third divisions were seen less frequently, especially in media without picloram and 1µM picloram, however both 0µM 2-ip-10µM picloram and 2µM 2-ip-10µM picloram media showed 100% of beads showing second divisions. Only 2µM 2-ip-10µM picloram media remained at 100% of all beads showing third divisions.

Much of the same pattern was repeated for peach palm protoplasts. The majority of all alginate beads embedded with peach palm protoplasts for all six treatments had signs of first cell division. All alginate beads for 0µM 2-ip-10µM picloram, 2µM 2-ip-1µM picloram, and 2µM 2-ip-10µM picloram treatments showed signs of first division and 91.7% of 0µM 2-ip-1µM picloram had cellular division. Only 66.6% and 58.3% of 0µM 2-ip-0µM picloram and 2µM 2-ip-0µM picloram beads, respectively, showed any sign of first cell division one week after protoplast isolation. The majority of beads for treatments with 1 or 10µM picloram showed second divisions, however there was a significant drop in beads showing third cellular divisions. Only 50% and 58.3% of 0µM 2-ip-10µM picloram and 2µM 2-ip-10µM picloram alginate beads showed signs of third cell division, compared to the comparably higher amounts for butiá cells.

Both peach palm and butiá had much more frequent signs of cellular division compared to açaí cells. The highest proportion of cells showing first cell divisions of embedded açaí protoplasts were the 2µM 2-ip-1µM picloram and 2µM 2-ip-10µM picloram treatments, each with 75% of

alginate beads showing active cell division. Bother these values are higher than the same levels of picloram without 2-ip, at 41.7% and 50% of beads showing first cell division, but this was only temporary. The proportion of cells showing second cell divisions was more similar, with 25% of 0µM 2-ip-1µM picloram and 2µM 2-ip-1µM picloram beads showing second cell division and 50% of 0µM 2-ip-10µM picloram and 66.6% of 2µM 2-ip -10µM picloram showing second cell division. Very few beads showed signs of third cell division, however the highest was 0µM 2-ip-10µM picloram and 201- at 25%. Compared to treatments with picloram, less than half of beads of 0µM 2-ip-0µM picloram and 2µM 2-ip-0µM picloram showed signs of first cell division. No further cell divisions were seen in 0µM 2-ip-0µM picloram and only 8.3% of 2µM 2-ip-0µM picloram beads showed second cell divisions. Petunia (*Petunia hybrida*) protoplasts were treated with different concentratins of the auxin 2,4-D and cytokinin BAP to evaluate the reentry of cells into the cell cycle. 2,4-D and not BAP was able to cause transcription of genes such as the mitogenactivated protein kinase (PMEK1), which might have further stimulated cell division (Trehin, et al., 1998).

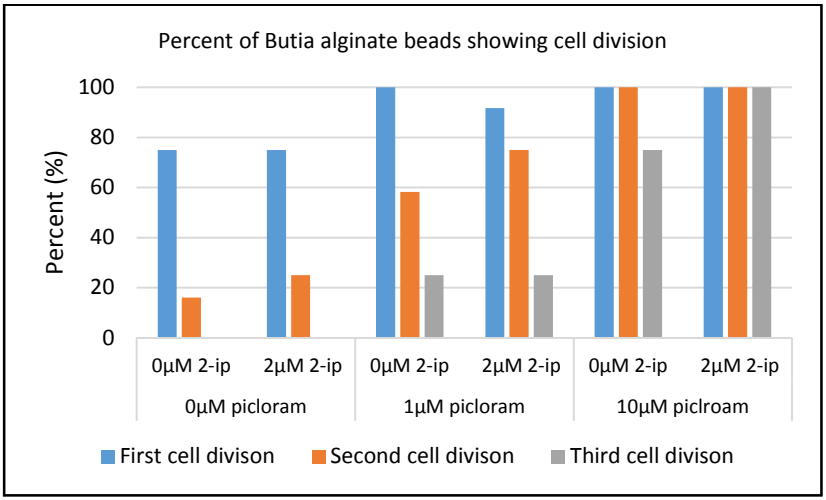

Figure 2-7: Percent of butia alginate bead showing protoplast cell division in liquid media containing different concentrations of growth regulators.

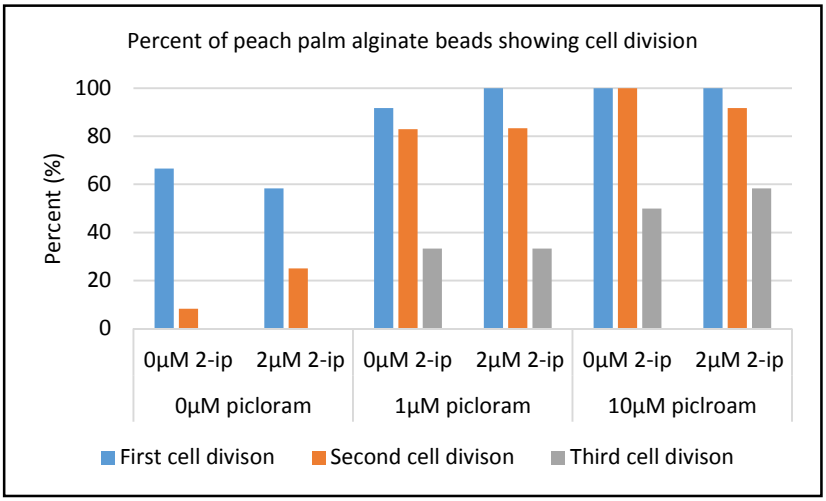

Figure 2-8:*Percent of peach palm alginate bead showing protoplast cell division in liquid media containing different concentrations of growth regulators.*

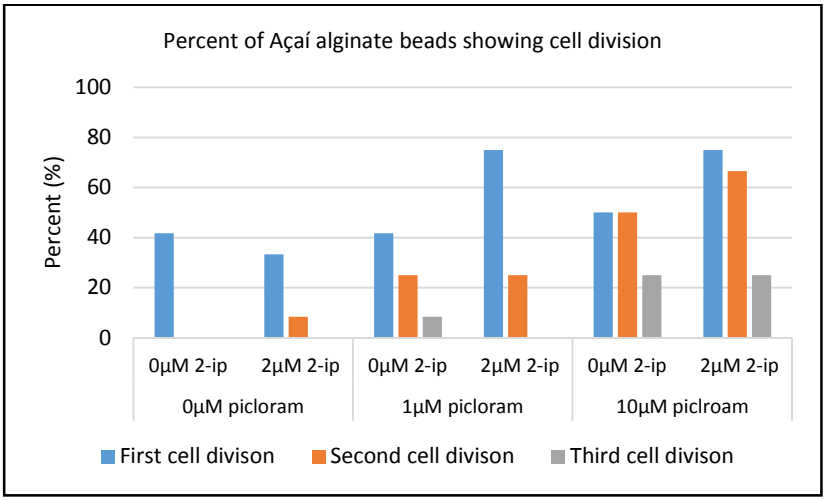

Figure 2-9: Percent of açaí alginate bead showing protoplast cell division in liquid media containing different concentrations of growth regulators.

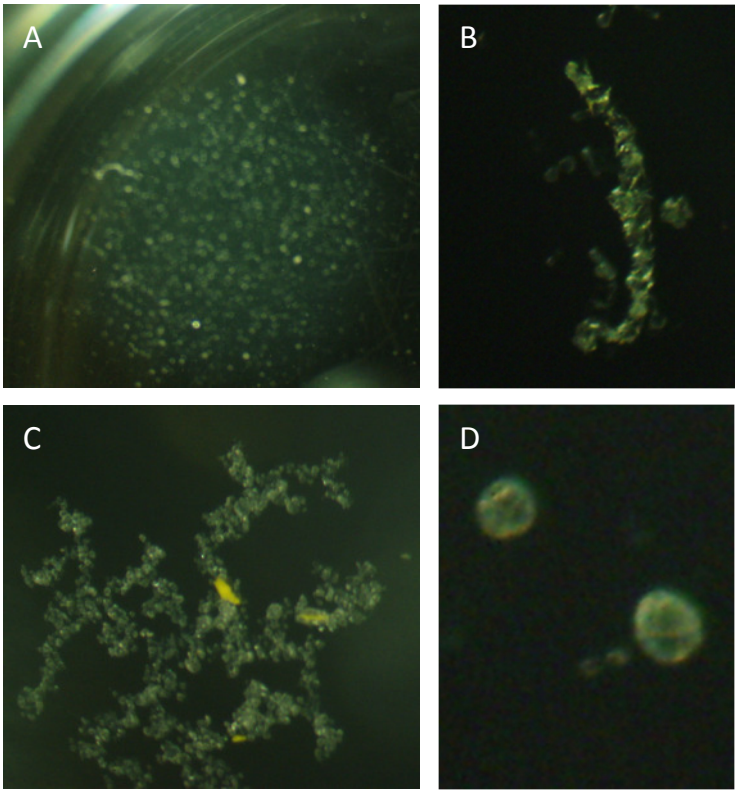

Figure 2-10: Regenerated peach palm microcalli. (A) Overhead view of growing microcalli embedded in 1.2% agarose; (B) Microcalli growing along a fiber; (C) Microcalli floating on the surface of the same media containing the agarose bead in A; (D) Small round regenerating structures found on the surface of the liquid media surrounding the agarose bead

Agarose beads

Preliminary experiments used embedding in agarose as the culture means, however, agarose was later substituted for alginate due to the general trend of improved regeneration in alginate systems over agarose described in the Davey et al., (2005), in addition to the possibility that the comparatively warm temperature of agarose might kill protoplasts. However, two agarose beads left in 2-10 media for about three months

did eventually grow microcalli (Figure 2-10). Microcalli were observed both embedded in agarose beads and floating on liquid media. However, further cultivation was made impossible due to contamination.

Long-term protoplast cultivation in alginate beads

Despite earlier success in cultivating peach palm microcalli using the agarose bead method, no microcalli have yet developed in alginate beads. Alginate beads have led to culture growth, including whole plantlet regeneration, in multiple *Daucus* species (Maćkowska et al., 2014), sweet orange (*Citrus sinensis*) (Niedz, 1993), and indian mango (*Mangafera indica*) (Rezazadeh et al., 2011). However, oil palm somatic embryos were regenerated using the agarose bead method (Masani et al., 2013). Additionally, these agarose beads were placed in liquid media contained in beakers on an orbital shaker, whereas alginate beads were left stationary. Future experiments should explore the use of stirring over stationary culture. Another source of optimization is culture density. Optimal cell densities were considered optimal between 1-2x10^5 cells/mL for white spruce (Attree et al., 1987), as well as 2-3x10^5 for *Citrus sinensis* (Castro et al., 2011). The same report found that 1x10^5 cells/mL, as used in this current study, was insufficient for achieving SE, but 5x 10^5 cells/mL promoted regeneration. Another major potential optimization is the use of nurse culture which were found in many species to promote protoplast regeneration such as in grape hyacinth (Karamian and Ranjbar, 2013), alfalfa (*Medicago sativa*) (Larkin et al., 1988), and three wild *Medicago* species (Gilmour et al., 1987).

Conclusion

Factors affecting protoplast isolation and culture from three Brazilian native palm species were investigated. Six hour incubation time was shown to yield the most peach palm and açaí protoplasts, however later incubation times led to large drops in protoplast yield. Twenty four hour orbital incubation time yielded the highest amount of butiá protoplasts. Shaking far preferable to stationary incubation, which yielded few protoplasts in comparison regardless of incubation time. Protoplast viability for peach palm and butiá remained stable and usually above 75%, but açaí cultures tended to lose viability with increasing shaking incubation time. Culturing protoplasts embedded in alginate was affected

by picloram concentration, which stimulated higher and earlier rates of cell division within the first week of culture. However, 2µM 2-ip had no obvious effect on early cell division. Exogenous piclroam should be considered essential for stimulating protoplast cell division for future experiments. Future studies should focus on the effect of culture density for stimulating culture growth. Higher concentrations of viable protoplasts cells may provide more effective protoplast regeneration. The widespread culture improvement through nurse culture is an important consideration for protoplast optimization. In addition, culture shaking vs. stationary culture could be another source of culture optimization. Little growth was seen in protoplast embedded in alginate under stationary conditions, despite the successful regrowth of peach palm microcalli embedded in agarose.

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3. Chapter 3: Biochemical profiles of high, low, and non-embryogenic peach palm 'Jirau 3' (*Bactris gasipaes*) cultures

Abstract

The biochemical profiles of highly embryogenic, low embryogenic, and non-embryogenic peach palm 'Jirau 3' (*Bactris gasipaes*) cultures was investigated. Samples were assayed for total soluble proteins, sugars, and starch and the results compared in terms of both fresh and dry weight. Low embryogenic cultures were composed of more water than either high or non-embryogenic cultures. After adjusting protein, sugar, and starch levels, the same trend was found, but the differences were not drastic. Highly embryogenic cultures contained slightly higher proteins content than non-embryogenic cultures, but both had significantly more protein content than low embryogenic cultures. Likewise, both high and non-embryogenic cultures had comparable levels of starch, but low embryogenic had less. All three cultures contained low levels of sugars as to be incalculable using a glucose standard curve, however, the absorbance readings at 620nm were significantly higher in both high and non-embryogenic cultures over low embryogenic cultures. These data suggest that although biochemical profiles of both high and non-embryogenic cultures are similar, their different morphologies and behavior are guided more through other factors, such as types of proteins being synthesized and genes expressed. Possible organogenesis of mesocarp tissue from non-embryogenic cultures based on biochemical profile, morphology, and color is also discussed.

Introduction

Though less cultivated than oil or date palm, peach palm (*Bactris gasipaes*), an Amazonian native palm, has potential commercial interest for sustainable heart-of-palm production due to its ability to regenerate multiple stems. In addition, the palm produces starchy fruits, although consumption is mainly isolated to areas in which it is cultivated (Mora-Urpí et al., 1997). Somatic embryogenesis (SE), the process of creating embryos from somatic cells, induction, culture multiplication, and embryo conversion has been thoroughly studied in this species in previous reports (Almeida and Kerbauy, 1996; Steinmacher, 2010; Steinmacher et al., 2007a, 2007b; Valverde et al., 1987). Peach palm, like most palms, is most effectively micropropagated using SE due to several reasons, including the ineffectiveness of vegetative propagation through low

rooting ability or due to only one single growing apex, as often seen in Juçara (*Euterpe edulis*) (Saldanha et al., 2006), recalcitrant seeds, such as in Chinese fan palm (*Livistona chinensis*) which prevent long-term storage (Wen et al., 2012), and many palm seeds have poor seed germination, such as in butiá-da-serra (*Butia eriospatha*) (Broschat, 1998). In comparison, (Abul-Soad and Mahdi, 2010) claim that 10,000 date palm (*Phoenix dactylifera*) plants had been regenerated from a single multiplied explant. This shows the importance of understanding this complex morphological pathway.

SE is the process by which a somatic cell dedifferentiates into an embryogenic state, divides, and begins to form a somatic embryo in morphological steps similar to zygotic embryogenesis (Verdeil et al., 1994). Embryogenesis entails widespread changes to gene expression, which is reflected in alterations to biochemical profiles, epigenetic state, and cell morphology. During zygotic embryogenesis (ZE), polyamine, auxin, abscisic acid (ABA), amino acids, proteins, starch, and overall dry matter were all found to gradually change during zygotic embryogenesis of loblolly pine (Pinus taeda) (Silveira et al., 2004). *Acca sellowiana* somatic embryo acquired protein and amino acid accumulation patterns similar to those found in zygotic embryos (Cangahuala-Inocente et al., 2014). Each biomolecule fulfills a key role that guides cell fate. Proteins provide biochemical and biophysical roles that aid in accumulation of reserves, maintaining active metabolism, and producing secondary metabolites. Sugars provide an energy source and carbon skeletons for amino acids and can provide energy reserves as starch.

While ZE and SE have nearly identical morphological routes, the biochemical changes are not as similar. Comparisons between date palm zygotic and somatic embryos showed that zygotic embryos contained higher overall proteins, many involved in storage and stress related functions, while somatic embryos had more proteins related to glycolysis and metabolism (Sghaier-Hammami et al., 2009). Indeed, accumulation of dry matter was observed over time in developing loblolly pine zygotic embryos, but somatic embryo dry weight did not increase (Pullman et al., 2003a).

Epigenetics, often measured in differences in overall global methylation, can play a large part in reconfiguration of a cell toward and embryogenic state (for review, see (Smulders and De Klerk, 2011). Comprehension of the biochemical changes between various types of tissue, such as highly embryogenic and non-embryogenic tissue, is essential to understand the mechanisms of SE. Therefore, the goal of this work was to compare overall protein, soluble sugar, starch, and dry eight to understand more about the role of nutrients in guiding cell fate toward being high, low, and non-embryogenic culture. Previous reports have explored the differences in amount of polyamines and several types of plant growth regulators, including ABA and IAA (Nascimento, 2012). IAA levels peaked during initial *Ocotea catharinensis* zygotic embryogenesis, suggesting a link between growth, cellular division, and establishment of bilateral symmetry essential for determining embryo polarity (Santa-Catarina et al., 2006). However, there is a limited amount of information on the biochemical profiles of different types of *in vitro* tissue, thus the general aim of this work was to deepen the understanding of SE in peach palm and help establish trends for markers of embryogenic competence.

Methods

Plant material

Peach palm cultures were previously established from excised zygotic embryos from fruits collected about 12 weeks after pollination from the peach palm germplasm bank of the Nacional de Pesquisas da Amazonia (INPA), Manus, Brazil and cultured as described in Heringer et al., (2014). Previously established peach palm cultures were routinely multiplied on Murishige and Skoog (MS) media composed of MS salts (Murashige and Skoog, 1962), Morel and Wetmore (MW) vitamins (Morel and Wetmore, 1951), 30g/L sucrose, 1g/L glutamine, and 10µM picloram. Highly embryogenic (HE) cultures consisting of rapidly dividing somatic embryos and proembryogenic masses were characterized by clusters of yellow, small, round, and smooth structures. Somatic embryos also display a suspensor region or are jointly connected to other somatic embryo through secondary somati embryogenesis. Low embryogenic cultures (LE) consisted of pale-yellow hard callus

displaying larger lobbed repetitive structures in circular or ridged arrangements which only occasionally gave rise to somatic embryosNon-embryogenic (NE) cultures consisting of softfibrous callus with a yellow colored exterior and white colored interior that, with very few exceptions, never gave rise to somatic embryos. All three tissue types were isolated and cultured separately (Figures 3-1 A-C).

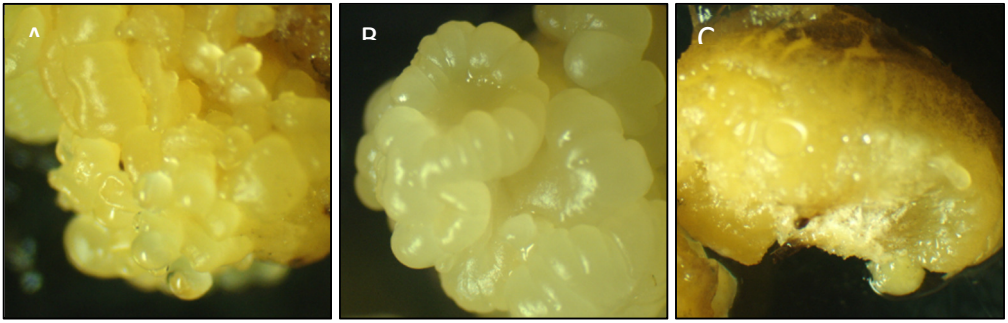

Figure 3-1: Types of tissues analyzed. (A) Highly embryogenic tissue was composed of nodular structures and somatic embryos. (B) Low embryogenic tissue was composed of large circular lobes often arranged in crescent or circular formations. Occasional isolated somatic embryos appeared on the outer edged, however small embryo clusters also appeared in the center of circular formations. (C) Non-embryogenic tissue had a white to pale yellow fibrous interior and often covered by a smooth yellow outer layer.

Dry Weight

Twenty 200mg samples of each culture type were excised, weighed and placed in a glass petri dish on a filter paper. The petri dishes were then incubated in an oven at 70°C overnight and remaining dried tissue was weighed again. The mean dry weight per tissue type was calculated using initial and final weights.

Protein measurement

Three hundred grams of fresh tissue from each culture was harvested and stored at -20°C for protein analysis. All steps were performed at 4°C to prevent protein degradation. Tissue was ground in liquid nitrogen in a chilled mortar and pestle and placed in 1mL of an extraction buffer (pH 7.0) containing 50mM sodium phosphate dibasic, 0.2M Beta-mercaptoethanol, 17.3 mM SDS, and 1mM PMSF. Samples were centrifuged for 20 mins at 8,300xg. The supernatant was removed and the

pellet was stored at -20°C. Soluble proteins in the supernatant were sedimented with two volumes of 100% ethanol at 0°C and then centrifuged at 12,850xg for 20 mins at 4°C. The protein sediment was solubilized in 50 mM sodium phosphate dibasic (pH 7.0) and protein content was determined according to Bradford (1976) with bovine serum albumin as the standard.

Total sugars

Soluble sugar extractions was done following Shannon (1968). The pellet from protein extraction was vortexed in 2mL of methanol-chloroform-water (12:5:3 v/v) and then centrifuged for 10 mins at 500xg. The supernatant was removed and stored. The pellet was then reextracted using 2mL of the same mixture and the supernatants combined. One part chloroform and 1.5 parts water were added for every four parts of the combined supernatants. The resulting solution was then centrifuged at 500xg for 10 mins. The resulting upper aqueous phase was removed and 0.2% w/v anthrone was added according to the procedure of Umbreit and Burris (1960). During early stages of Acca sellowiana somatic embryogenesis, sugar and starch concentrations increased during the heart and cotyledonary stages, but decreased during the torpedo and cotyledonary stages (Cangahuala-Inocente et al., 2009).

Starch measurement

Starch extractions was performed according to McCready et al., (1950). The pellet from total sugar extractions was ground in 1mL of 30% perchloric acid and centrifuged for 15 mins at 12,850xg. The supernatant was removed and the pellet re-extracted again. The supernatants were combined and the pellets were discarded. Both sugar and starch concentrations were read in a UV= VIS UV-1203 spectrophotometer at 620nm using glucose as a standard.

Data analysis

Data was analyzed by ANOVA with additional Tukey tests (alpha = 0.05). All tests were performed a minimum of six times. For comparisons between total sugars, protein, and starch, a general analysis with all six replicates was performed, however, there was large variation. Within each tissue type, the mean was found and the two data points with the highest

residuals were eliminated and the analysis performed again. Data for total sugars, proteins, and starch were reported in terms of fresh weight, however, dry weight was found to vary significantly between each tissue type. The amount of sugars, starch, and proteins were analyzed in terms of dry weight as well by dividing one by the percent of dry weight as a decimal. The resulting number was then multiplied to each data point to find the amount of sugar, starch and protein by gram dry weight.

Results

Dry weight

Bother HE and NE cultures had comparable water content, however low embryogenic cultures had higher average water content (Table 3-1). The calculated dry weight for HE, LE, and NE cultures were 9.62±0.74%, 7.94±0.46%, and 9.32±0.57%, respectively. When dry weight was accounted for, the multiplication factors for calculating protein, starch, and sugar levels in terms of mg/g DW were 10.39 (HE), 12.6 (LE), and 10.73 (NE). There was no significant difference between HE and NE dry weights. These findings conflict with (Neves et al., 2003), who characterized non-embryogenic sugarcane (*Saccharum sp.*) callus as having a lower dry matter/fresh matter ratio than embryogenic callus. Light squeezing with forceps reveled that large amounts of water could be released for low embryogenic cultures, but not HE and NE cultures. HE tissue was hard and compact, whereas NE tissue was soft and fibrous. Due to these differences in water content, further analysis was done in light of both FW and DW. The high levels of water found in HE tissue, which included somatic embryos, would be expected to diminish as maturation took place during natural embryogenesis, as demonstrated in the initially high water content of Nihoa fan palm (*Pritchardia remota*) zygotic embryos gradual reduction as reserve compounds were (Pérez et al., 2012). However, as found in loblolly pine (Pinus taeda), somatic embryos might retain high levels water content in comparison to zygotic embryos (Pullman et al., 2003a). Future studies may investigate dry weight accumulation in mature peach palm somatic embryos during various stages of SE with the same biochemical studies to evaluate if the reserve compounds required for SE maturation and conversion are accumulating.

Table 3-1: Biochemical compositions of peach palm 'Jirau 3' tissue with varying embryogenic potential.

Tissue type	Dry weight (%)	Soluble proteins (mg/g) Column1		Starch (mg/g) Column2		Soluble sugars (absorbance at 620nm) Column3	
		FW	DW	FW	DW	FW	Absorbance x DW
Highly Embryogenic	9.62±0.74% A	0.161±0.005 A	1.67±0.056 A	26.92±5.07 A	279.67±52.64 A	0.150±0.006 A	1.56±0.064 A
Low Embryogenic	7.94±0.46% B	0.088±0.006 B	1.11±0.070 B	8.33±2.40 B	105.0±30.22 B	0.082±0.009 B	1.039±0.120 B
Non-Embryogenic	9.32±0.57% A	0.137±0.013 A	1.47±0.142 AB	24.14±2.50 A	258.83±26.88 A	0.139±0.009 A	1.487±0.100 A

1Capital letters designate significance based on Tukey statistical analysis (alpha = 0.05)

Protein amounts

No significant difference in total protein content was found between HE and NE cultures, however, HE showed almost double concentrations of proteins per gram FW compared to LE cultures (Table 3-1). However, when compared in terms of DW, the differences between HE and NE become less apparent, with 1.67±0.056 mg protein/g DW for HE and 1.47±0.142 mg protein/g DW for NE. Even after converting into terms of DW, LE protein levels remained lower than either HE or NE at 1.11±0.070 mg protein/g DW. Despite the large morphological differences, there was not a major difference in protein concentration between HE and NE cultures. This is in contrast to comparisons between rice (*Oryza sativa*) embryogenic cultures containing 1.6 times more protein than non-embryogenic cultures (Chen and Luthe, 1987). Rather, the types of proteins between these two types of tissue may show clear differences. Comparisons between arabinogalactan proteins found on embryogenic and non-embryogenic *Euphorbia pulcherrima* callus cultures found that both types of tissue contained different sets of arabinogalactan proteins (Saare-Surminski et al., 2000). Embryogenic and non-embryogenic saffron (*Crocus sativus*) cultures accumulated different sets of proteins, such as higher numbers of proteins associated with regulating oxidative stress in embryogenic cultures over non-embryogenic cultures (Sharifi et al., 2012). LE tissue might be simply

water logged tissue that expends all of its energy growing as a callus mass, rather than develop an abundance of many types of proteins to create more complex tissue.

Starch amounts

LE tissue was found to have low starch levels at 8.33±2.40 mg/g FW compared to either HE (26.92±5.07 mg/g FW) or NE (24.14±2.50mg/g FW). Even when compared after accounting for DW, either tissue contained significantly higher levels of starch. For every gram of dry weight, 27.9±5.64% of HE, 10.5±3.02% of LE, and 24.8±2.69% of NE tissues are composed of starch, a significant part of dry mass. Starch reserves may fuel future rapid growth, however, both NE and HE had large amounts compared to LE tissues. This is in contrast to higher starch levels found in non-embryogenic alfalfa cultures compared to embryogenic cultures (Martin et al., 2000b). However, both increased protein and starch levels were associated with improved peach palm multiplication in a RITA bioreactor (Heringer et al., 2014). Starch reserves were found to increase during early oil (Kanchanapoom and Domyoas, 1999) and date palm (Sghaier et al., 2009) embryo formation. During maturation, these starch reserves were found to rapidly disappear in embryo maturation (Gomes et al., 2014). Starch hydrolysis provides large amounts of metabolic energy for cell division and formation of specialized tissues (Martin et al., 2000b). Interestingly, a comparison between grape 'Grenache noir' (Vitis vinifera) zygotic and somatic embryos found that although both acquired accumulated starch and lipid reserves, somatic embryos were unable to use storage compounds due to an absence of isocitrate lyase (Faure and Aarrouf, 1994).

Sugar amounts

Differences were detected between absorbance readings at 620nm, however, the absorbance readings were low as to have negative values in terms of mg/g FW using the glucose standard curve. However, LE tissue had significantly lower absorbance values compared to either HE or NE tissues. HE tissues had slightly higher absorbance values than NE tissue, but these values were not significant. When compared after multiplying the absorbance ratings by the DW multiplication factor, the differences between either HE and NE became less apparent. However, LE

absorbance values remained significantly lower than either tissue type. No difference in total sugars was detected between embryogenic and non-embryogenic alfalfa cultures, although embryogenic cultures contained higher levels of sucrose and non-embryogenic calli (Martin et al., 2000a). High levels of reducing sugars and low levels of phenols were detected in cotton (*Gossypium spp*.) embryogenic cultures compared to non-embryogenic cultures (Obembe et al., 2010). Embryogenic cacao (*Theobroma cacao*) cultures contained higher cysteine as well as reducing sugars compared to non-embryogenic cultures (Emile et al., 2013). Higher total soluble and reducing sugars were detected in embryogenic banana (*Musa spp*.) cultures compared to non-embryogenic cultures. Additionally, embryogenic cultures contained higher IAA, ABA, and zeatin riboside, while non-embryogenic contained higher levels of gibberellic acid (Wang et al., 2013).

Tissue types and possible organogenesis

These data presented here shows the complexity of *in vitro* culture biochemistry. Despite the same concentrations of soluble proteins, both NE and HE might contain different sets of proteins, as found in a proteomic comparison of maize (*Zea mays*) embryogenic and non-embryogenic cultures (Sun et al., 2013). The similar results in starch reserves and sugar absorbance ratings might indicate that these tissues may not vary in terms of overall amounts, but rather on the epigenetic and genetic state of the tissue. Differences between embryogenic and non-embryogenic culture biochemical profiles have been previously reported in peach palm cultures, such as higher total phenolics and polyamines, especially putrescine, in non-embryogenic peach palm tissues and higher IAA and ABA concentrations in embryogenic cultures (Nascimento, 2012). An important distinction between the tissues is morphology. Frequently, NE tissue took on a rounded smooth shape with visible fibers and deep yellow color. Some peach palm varieties have fruits with starchy fibrous mesocarps which can range from yellow to orange in color. The large stores of starch and morphology could indicate that certain kinds of callus are actually other organogenic structures, specifically mesocarp. Mesocarp can grow in response to tissue culture environment, including

presence of auxin, as shown by peach (*Prunus persica*) mesocarp discs were treated with several levels of NAA with the result that auxins promoted both disc enlargements and ripening processes, such as mesocarp softening and anthocyanin formation (Ohmiya, 2000). LE tissues were often composed of lobbed structures which curled inward toward the center of the growing callus. The same structure was seen in long-term areca palm (*Areca catechu*) cultures, although it was hypothesized that these were aberrant somatic embryos (Wang et al., 2010). Somatic embryos grow in clusters or singly away from the center of the culture without pattern, whereas the lobbed structures grew in a circular or crescent pattern. It is therefore possible that the lobbed structures are another form of organogenesis. However, this would be difficult to verify. Much of LE tissue is composed of large masses of callus tissue, which were analyzed in conjunction with the lobbed structures. The low levels of proteins and starch, as well perhaps the lower sugar mean absorption levels, could indicate that reserves are not being directly synthesized, rather all chemical energy is directed toward cell division. The end result is a large mass of tissue with high fresh weight, but low dry weight. Both HE and NE tissues have acquired reserve compounds and thus display that different biochemical processes are active. No known report of *in vitro* mesocarp organogenesis has been published, but the organized tissue morphology, presence of yellow secondary pigment metabolites, fibers, and biochemical might indicate that more tissues other than callus and somatic embryos are being produced by 'Jirau 3' peach palm cultures.

Future studies may focus on a more comprehensive biochemical analysis involving concentrations of auxins, amino acids, and polyamines, as well as a more thorough morphological analysis of the distinct structures found growing along with somatic embryos.

Conclusion

Somatic embryogenesis and *in vitro* tissue development is a complex system that is dependent on a wide variety of biomolecules. The results presented here present many conflicting observations to other studies of embryogenic against non-embryogenic tissue, especially the finding of similar levels of starch and protein between high embryogenic and non-

embryogenic cultures. Low embryogenic cultures displayed lower protein, starch, sugar, and overall lower dry weight compared to high embryogenic and non-embryogenic cultures, both of which were comparable for all four factors. The major differences between the tissue types might not be highly influenced by total amounts of biomolecules, but rather what genes are being expressed, concentrations of plant hormones, and specific types of proteins being synthesized. The possibility of multiple types of tissue being produced is possible due to organized tissue structures, which might result in entirely different biochemical profiles. However, these possibilities would require a more in-depth study to validate.

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4. Chapter 4: Attempts to Induce Somatic Embryogenesis in Recalcitrant Butiá-da-serra (*Butia eriospatha*), Improve Somatic Embryo Multiplication in Peach palm (*Bactris gasipaes*), and Improved Somatic Embryogenesis in Açaí (*Euterpe edulis*) through Combination of Activated Charcoal and Increased Picloram

Abstract

Optimization of somatic embryo multiplication is essential for providing efficient palm micropropagation. However, there are many possible approaches that can be used to improve somatic embryogenesis induction and culture multiplication. The present work covers several varied approaches to improving somatic embryogenesis in three native Brazilian palm species: peach palm (*Bactris gasipaes*), açaí (*Euterpe oleracea*), and butiá-da-serra (*Butia eriospatha*). Peach palm cultures were investigated for the effect of modified MS media, mixed carbohydrate source, partial desiccation, and polyamine addition on somatic embryo multiplication. There was no significant difference between experimental treatments and control, except on the latter in which it was found that 1µM spermine or spermidine had a negative effect on number of embryos produced by peach palm cultures. Açaí tissue multiplication was greatly improved by addition of 2.5g/L activated charcoal with 50µM picloram through lower tissue oxidation, increased proportion of explants with embryos, increased embryos per explant, increased proportion of explants with polyembryogenic masses, and increased numbers of polyembryogenic masses per explant. However, increased callogenesis and organogenic root formation was also observed in açaí cultures on MS media with activated charcoal and 50µM picloram. Somatic embryogenesis was not induced on friable butiá cultures in a large variety of different media and treatments, however proembryogenic structures were induced on butiá zygotic embryos placed on MS media with 300 or 450µM picloram.

Introduction

Palm micropropagation

Efficient and reliable palm micropropagation is essential for supplying elite cultivars to farmers, generating more individuals of threatened species, and providing predictable means of culturing plants of scientific interest. To this end, protocol improvement not only increases efficiency of *in vitro* regeneration, but also helps establish trends which can be followed to predict success in other genotypes. Somatic embryogenesis (SE), the process by which embryos are regenerated from somatic cells through cell dedifferentiation and regeneration through morphological

steps similar to zygotic embryogenesis, is the most widely used route to creating new palms (Verdeil et al., 1994). This route entails vast changes in gene expression (Singla et al., 2007), reorganization of epigenetic state (Smulders and De Klerk, 2011), increased metabolism to fuel continued cell division (Lipavská and Konrádová, 2004), and differentiation of specialized tissues (Moura et al., 2010). Tissue culture media, at the bare minimum, must contain macro and micro nutrients, a carbon source which can also confer osmotic pressure, such as sucrose, and auxins such as 2,4-D or picloram. Auxins were found to be essential in multiple palm species, including sago palm (*Metroxylon sagu*) (Alang and Krishnapillay, 1987), coconut (*Cocos nucifera*) (Karunaratne and Periyapperuma, 1989), and oil palm (*Elaeis guineensis*) (Guedes et al., 2011). However, recalcitrance to regeneration is a constant obstacle for SE in many species, but media optimization can improve somatic embryo induction, multiplication, maturation, and conversion.

Palms

Açaí (*Euterpe oleracea*) is an Amazonian palm that is most known for its fruit pulp, which can be eaten fresh or added to beverages, and heart-of-palm, the soft immature leaves eaten pickled or fresh as a vegetable. Cultures had already been established in our lab, however, the first account of SE in açaí was reported by Ledo et al., (2002) using zygotic embryos. Current cultures in the lab consist of large groups of small white somatic embryos, often connected by irregular tubular structures, potentially serving as organogenic inflorescences. However, despite the relative ease of stimulated SE, the cultures tended to oxidize rapidly, thus requiring two-week subcultures to prevent culture death.

Peach palm (*Bactris gasipaes*) is a caespitose palm native to the humid Amazon tropical rainforest (Mora-Urpí et al., 1997). Long before the European arrival to the Americas, Amazonian tribes made extensive use of peach palm fruit as a source of starchy fruit for food, timber for building, and leaves for baskets and clothing (Mora-Urpi et al 1997). Currently, it is still used agriculturally as a source of starchy fruit and heart-of-palm (Clement et al., 2004). Peach palm has been highly studied

in our lab (Heringer et al., 2013; Nazário et al., 2013; Steinmacher et al., 2007a).

Butiá, or butiá-da-serra (*Butia eriospatha*) is a native Brazilian palm native to the Mata Atlantica biome (Minardi et al., 2011). The palm produces a sweet fruit, which can be eaten fresh, made into jelly, used as a flavoring in confections, or placed in bottles to flavor liquor. Butiá cultures were previously induced using the horizontal thin cell method on apical meristems, resulting in fast growing friable yellow callus. However, despite the relative ease of multiplying this callus, it remained highly recalcitrant to inducing SE. Currently, there are no reports of successful SE induction in *Butia eriospatha*, nor indeed any *Butiá* species.

Activated charcoal

One of the most common culture additions, especially during initial culture induction, is activated charcoal (AC) because of its action in preventing rapid tissue oxidation. The exact mechanism AC performs *in vitro* is not completely understood, but it may be related to antioxidant behavior in minimizing the effect of radicle oxidative species (ROS), providing a dark environment, release of growth-promoting substances, absorption of harmful substances, or modifying growth regulator effects (Weatherhead et al., 1978). AC reduces the oxidative stress of ROS, such as hydrogen peroxide and superoxide, which can lead to tissue darkening and culture failure (Apel and Hirt, 2004). High levels of ROS in cells may lead to protein denaturation, peroxidation of unsaturated fatty acids, and damage to nucleic acid (Blokhina et al., 2003). A common observation is the interference AC has on auxin signaling by requiring increased concentrations of exogenous auxin in order to have the same effect (Pan and Staden, 1998). In addition to auxin, AC was seen to be essential in several species, such as macaw palm (Moura et al., 2009) Previously-established açaí cultures tended to rapidly oxidize if not frequently subcultured onto new media every two to three weeks, therefore, activated charcoal and increased picloram were investigated as a means of improving somatic embryo multiplication without rampant tissue oxidation.

Carbohydrate source

The role of carbohydrates is not limited to energy source, but make up carbon skeletons for amino acids, structural components, relocated into lipids, signal molecules, or other ways carbon is incorporated into cellular structure (Yaseen et al., 2013). In order to generate the large numbers of types of biomolecules, the cell requires an active metabolism and a constant supply of fuel. Carbon sources other than sucrose and glucose, such as mannose, maltose, galactose, and glycerol, may be potential alternatives (Beyl, 2011). The sugar alcohol sorbitol has also been shown to be a viable carbon source and causing osmotic stress to disrupt plasmodesmatal connections (Yaseen et al., 2013), thus isolating cells and aiding in cell dedifferentiation. Despite the role of various carbohydrates in bioprocesses, no work has been published on using mixtures of two different carbohydrate sources, although comparisons between individual carbohydrate sources have been tested, such as sucrose and glucose (Duncan et al., 1985). However, a study in SE in loblolly pine suggests that by mimicking the carbon sources found in naturally found seeds, SE is improved (Pullman et al., 2003b). Therefore, a carbohydrate mixture was of interest for improving peach palm somatic embryo multiplication.

Basal media

Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) is the most commonly used basal salt mixtures used in palm SE. MS salts have been shown to be a functional basal media in numerous plants, such as poplar (*Populus tremula*) (Huang and Dai, 2011), cocoa (*Theobrama cacao*) (Esan, 1992), and maize (*Zea mays*) (Conger et al., 1987), although composition of macro and microelements is an often over-looked variable. For example, MS media contains high concentrations of ammonia, which can accumulate in plants under certain conditions (Lacuesta et al., 1997), thus causing a futile cycle in which ammonia enters the cell, where it can either be processed through the conversion of glutamate to glutamine or be cycled out of the cell at the cost of ATP, thus potentially hampering growth (Szczerba et al., 2008).

Other basal salts have been tested in a large number of palm species. Indeed, N6 salts (Chu, 1978) showed improved regeneration in oil palm over MS salts Eeuwen's Y3 media (Eeuwens, 1976) (Padilha, 2013) had comparatively improved SE induction on coconut zygotic (*Cocos nucifera*) (Gupta et al., 1984) embryo and jungle salak (*Salacca glabrescens*) (Zulkepli et al. 2011) over MS media. To predict peach palm tissue reaction to various levels of macro and micro elements, growth responses to fertilizer from several studies were analyzed. Among the results of different fertilizers was a distinct trend of magnesium and boron shortage (Ares et al., 2003; Schroth et al., 2002) and suggestions of medium levels of nitrogen fertilizer (Deenik et al., 2000). However, the effect of combined field fertilizer studies and *in vitro* trends in other palms was of interest to devising a new optimized media for peach palm cultures.

Polyamine addition

Polyamines are low molecular eight polycations essential for growth in both prokaryotic and eukaryotic cells (Kaur-Sawhney et al., 2003). These molecules stimulate DNA replication, transcription, and translation and, therefore, are of interest for SE (Silveira et al., 2006). In several studies, addition of polyamines, such as spermine and putrescine, have promoted in holm oak (*Quercus ilex*) and Scots pine (*Pinus sylvestris*) (Mauri and Manzanera, 2011; Vuosku et al., 2012). In oil palm, 100 µM of spermine and 1mM putrescine led to greater numbers of embryogenic calli, somatic embryos, and embryo conversion over control without polyamines (Rajesh et al., 2003). Therefore, the effect of exogenous polyamines was tested on peach palm cultures in order to evaluate if culture multiplication could be improved.

Partial dehydration

Othmani et al., (2009) used partial desiccation of date palm calli over several intervals to vastly increase the number of somatic embryos regenerated. Partial dehydration also increased organogenesis in Oryza japonica rice, leading to greatly enhanced numbers of regenerated

plantlets (Rancé et al., 1994) Additionally, partial desiccation of Interior spruce (*Picea glauca x P. engelmannii*) (Roberts et al., 1990) and English walnut (*Juglans regia*) (Tang et al., 2000) greatly increased the rate of somatic embryo conversion (Tang et al., 2000). There is little study of the effect of partial desiccation on somatic embryo multiplication, therefore both peach palm and butiá cultures were placed in partial desiccation treatments in order to evaluate the effect on SE.

Liquid media

Liquid media has been shown to promote SE in several species over solid media, as shown through suspension cultures in date palm (Fki et al., 2003). Oil palm suspension cultures from friable embryogenic calli and placed into regeneration media, which developed into mature somatic embryos, which were then converted into plants (Teixeira et al., 1995). Current butiá cultures are friable and well adapted to making suspension cultures, however they are continuously kept on solid multiplication media. Therefore, the effect of culturing in solid vs. liquid media with or without growth regulators was investigated. RITA temporary immersion bioreactors were found to have a positive effect on peach palm somatic embryo culture (Heringer et al., 2014), therefore the success was attempted to be replicated using açaí somatic embryos.

Abscisic acid

ABA is a common plant growth regulator heavily involved in inducing embryo maturation and preventing precocious germination (for review, see (Rai et al., 2011). ABA was instrumental in the acquiring of reserve compounds (Sghaier-Hammami et al., 2010) and increasing dry weight (Sghaier et al., 2009). Both ABA 20 or 40µM and 90g/L sucrose for 2-4 weeks was sufficient to increase protein content within date palm somatic embryos (Sghaier-Hammami et al., 2010). Sugar cane (*Saccharum sp*p.) somatic embryo treated with 3.8uM ABA were shown to have increased proteins, polyamines, proline, and starch (Nieves et al., 2001). In addition to its role in maturation, ABA increased the number of somatic embryos in date palm (Zouine et al., 2005) and coconut (Fernando and Gamage,

2000). Exogenous 2,4-D and ABA was beneficial for maize (*Zea mays*) somatic embryo proliferation (Emons et al., 1993). The role of ABA was investigated in several ways. First, exogenous ABA was of interest to butiá cultures in order to potentially induce somatic embryo differentiation.

Gibberellic acid

Gibberellic acid, often referred to as GA3, is a plant hormone highly related to embryo germination and wide changes to gene expression (Schwechheimer and Willige, 2009). Notably, GA3 has shown to demonstrate its use in tissue culture by increasing conversion rates of somatic embryos, as demonstrated in Siberian ginseng (*Eleuterococcus senticosus*) (Chakrabarty et al., 2003) or in encapsulated *Citrus reticulate* somatic embryos (Antonietta et al., 1998). However, GA3 addition to media increased the number of coconut calli that formed somatic embryos by 50% and nearly doubled the number of somatic embryos (Montero-Córtes et al., 2010), thus showing that GA3 might have an additional role in SE. In the same report, expression of a KNOTTED-like homeobox gene was stimulated by GA3 and related to SE, but its expression was not detected in aberrant embryos. Gibberellins, antagonists of ABA, are known to be involved in germination, thus causing widespread changes to gene expression and metabolism (Hochholdinger et al., 2006). Therefore, the effect of GA3 was of interest both for potential use in stimulating SE in butia.

PGR-free media

Absence of plant growth hormones (PGR) has shown to be beneficial for stimulating SE in otherwise recalcitrant species. Chinese cotton (*Gossypium hirsutum*) somatic embryos were differentiated from embryogenic calli by placing calli on PGR-free MS media with high nitrate ,eventually leading to globular embryos (Kumria et al., 2003). Recalcitrant chinese cotton cultures were success fully induced to form somatic embryos using media without growth regulators and doubled potassium nitrate (Wu et al., 2004), but decreasing maconutrient

concentration alleviated culture browning (Wang et al., 2006). In order to attempt to stimulate SE in butiá, PGR-free media with or without 2.5g/L AC and full or half MS macronutrients with or without doubled potassium nitrate were treatments of interest.

Goal

The goal of the present work was to investigate little-explored methods of improving somatic embryogenesis, as well as induce somatic embryogenesis in recalcitrant butiá cultures.

Methods

Plant material

Peach palm cultures were previously established from excised zygotic embryos from fruits collected about 12 weeks after pollination from the peach palm germplasm bank of the Nacional de Pesquisas da Amazonia (INPA), Manus, Brazil and cultured as described in Heringer et al., (2014). Butia-da-serra and acai apical meristems were harvested from one to two year old immature palms and cultured according to the thin cell layer method described in Steinmacher et al., (2007c). Peach palm cultures were continuously multiplied on medium consisting of Murishige and Skoog (MS) salts (Murashige and Skoog, 1962), Morel and Wetmore vitamins (MW) (Morel and Wetmore, 1951), 30g/L sucrose, 1 g/L glutamine, 10µM picloram and solidified with 2.3g/L phytagel. Highly embryogenic, low embryogenic, and non-embryogenic tissue were separated and placed in separate petri dishes during monthly subcultures. Butiá cultures were initiated using thin cell layers placed on MS medium with MW vitamins, 30g/L sucrose, 300µM picloram, and 2.3g/L phytagel until a friable yellow calli formed. The callus was divided and multiplied on multiplication media. Açaí cultures were originally multiplied on multiplication medium until it was found that 50µM picloram and 2.5g/L AC were shown to be beneficial. Subsequent experiments with açaí used this modified multiplication media. Açaí on normal multiplication media were subcultured at biweekly intervals to limit tissue oxidation. After changing to 50µM picloram and 2.5g/L modified multiplication media, cultures were subcultured at monthly intervals. All media had their pH

adjusted to 5.8 using 1M NaOH before being autoclaved. All cultures were placed in a dark growing chamber at 25 +/-2 °C unless otherwise noted.

Peach palm somatic embryo multiplication studies

New basal mixture, carbohydrate source, polyamine addition, and partial dehydration peach palm studies: One gram of highly embryogenic peach palm tissue was placed on either normal multiplication media or multiplication with the substituted new basal media salt mix (table 4-1), carbohydrate mix (Table 4-2), 1µM spermine or 1µM spermidine, or first partially dehydrated by first placing cultures in a sterile petri dish with a sterile filter paper for 0, 6, 12, or 24 hours before being placed on multiplication media. Cultures were left to grow for a month the number of recovered somatic embryos were counted. Basal media and polyamine experiments were conducting using 'G3' peach palm culture line, the carbohydrate study used 'G2' peach palm tissue line, and partial dehydration used "Jirau 3' high and low embryogenic cultures.

Table 4-1: Composition of MS salts and modified MS salts tested on peach palm embryo multiplication

	MS (mg/L)	Modified MS (mg/L)
NH4NO3	**1650**	**0**
(NH4)2SO4	**0**	**300**
KNO3	**1900**	**2500**
KH2PO4	**170**	**340**
MgSO4	**180.7**	**300**
CaCl2	332.2	332.2
KI	0.8	0.8
H3BO3	**6.2**	**10**
MnSO4	16.9	16.9
ZnSO4	8.6	8.6
CoCl2	**0.025**	**0.05**
CuSO4	**0.025**	**0.05**
Na2MoO4	0.25	0.25
FeSO4	27.8	27.8
Na2EDTA	37.2	37.2
mg/L	**4330.7**	**3870**

Table 4-2: Carbon sources used for peach palm somatic embryo multiplication

	Sucrose	Fructose	Glucose	Sorbitol
Molecular weight	342.3	180.16	180.16	182.17
Control concentration (M)	**0.087642**	0	0	0
Control (g/L)	30	0	0	0
Mixture concentration (M)	**0.021911**	**0.0219**	**0.0219**	**0.0219**
Modified mixture (g/L)	7.5	3.945504	3.95	3.989523

Açaí somatic embryo multiplication studies

Açaí cultures were placed onto either multiplication media with 10 µM piclroam (P10) or modified multiplication media with 2.5g/L AC with 50µM picloram (P50AC). Cultures were left to grow for a month before percent of cultures showing 50% or greater tissue oxidization, percent of explants with embryos, number of embryos per explant, percent of explants with polyembryogenic masses (PEMs), number of PEMs per explant, percent of explants with callus, and percent of explants with organogenic growth were counted (Figures4-1 A-F).

Half a gram of açaí cultures were placed either on solid p10 media, submerged in 50mL of liquid P10 media in a 250mL Erlenmeyer flask and placed on an orbital shaker at 90 rpm, or placed in a RITA® bioreactor with p10 media with 3 minute immersion times every 3 hours.

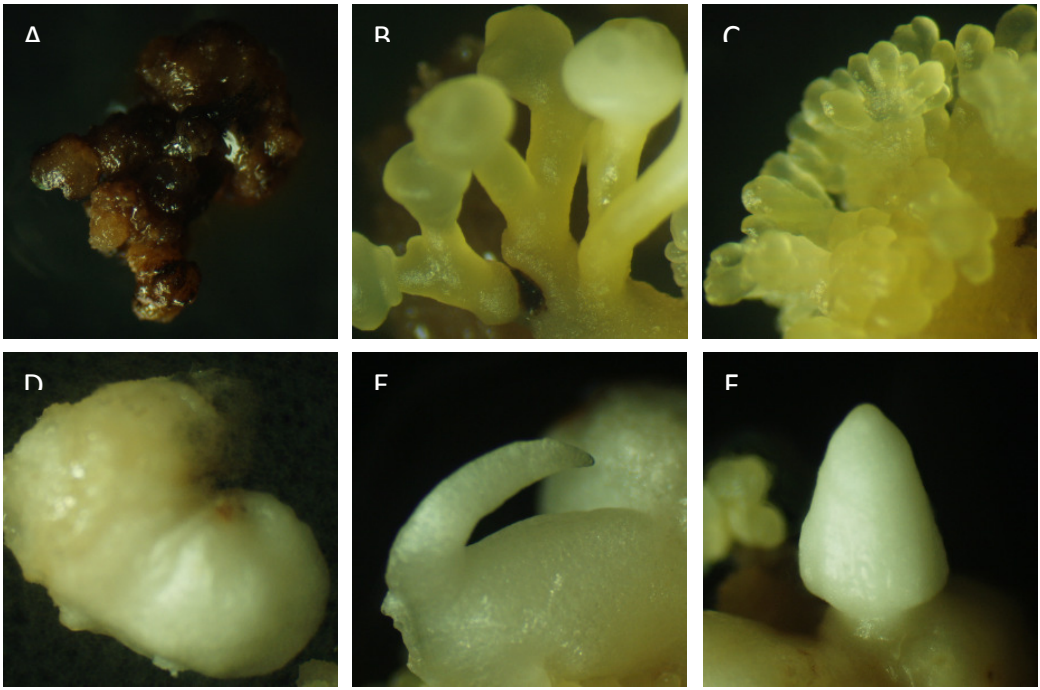

Figure 4-1: Types of resulting tissue from açaí multiplication in either p10 or p50AC media. (A) Oxidized explant; (B) Somatic embryos; (C) Polyembryogenic mass; (D) Callus; (E) Organogenic root; (F) Mature somatic embryo

Butiá somatic embryo induction studies

Butiá cultures were placed on several types of media in order to stimulate SE (see table 4-8 for a complete list). Butiá were either left on multiplication media or transferred to multiplication media with 14.7µM 2-ip, no picloram, and 5µM NAA with or without 5µM ABA and placed in either the light or dark. Cultures were placed on media with or without 1µM GA3 and 3 or 8% sucrose. Other cultures were also placed on MS media without GR with half, full, or double potassium nitrate and placed either in the light or dark. To evaluate if partial desiccation had a beneficial effect on inducing SE, cultures were placed on autoclaved filter paper n a petri dish and left for 12, 24, or 48 hours before being removed

and placed on multiplication media. To evaluate the effect of fine chopping, cultures were removed and chopped using a sharp scalpel before being spread out on multiplication media. The effect of liquid media vs. solid media for differentiation was investigated by culturing 1g of butiá callus onto solidified multiplication media, into 50mL of liquid MS media in a 250mL beaker, onto solidified MS media without growth regulators, or into 50mL of liquid media MS media without growth regulators.

Butiá zygotic embryo induction

Butiá seeds were washed in 2% sodium hypochlorite with one drop of tween 20 per 100mL of solution for fifteen minutes before being rinsed three times with distilled autoclaved water. Embryos were excised and placed on 10mL of media containing MS media, MW vitamins, 3% sucrose, 1g/L glutamine, 2.5g/L AC, 2.3g/L phytagel, and 0, 300, or 450µM picloram in test tubes. Two zygotic embryos were placed in each test tube with each tube considered a replicate. Cultures were placed in the light and left without subculture for twenty weeks. At the end of twenty weeks, the number of zygotic embryos that grew callus, germinated, oxidized, or failed to respond were counted.

Statistics and data analysis

The basal salt mixture test, polyamine addition test, carbohydrate mixture test, partial dehydration tests were carried out with three replications. The modified multiplication media test was carried out with 14 replications consisting of 30 pieces of açaí culture added to both normal multiplication media and modified media from a one-month old açaí culture. All butiá tests were carried out with three replications. All appropriate tests were analyzed using ANOVA with a SNK test.

Results

Açaí test

Açaí culture multiplication was dramatically improved by placing explants on MS media containing 50µM picloram and 2.5g/L AC over multiplication media with 10µM picloram alone (Table X). Explant oxidation was significantly reduced from 27.9±5.24% of p10 explants showing 50% or greater oxidation to 6.99±3.82% for the P50AC media

($p<0.001$). This likely led to p50AC media showing greater numbers of explants with embryos at 88.6±4.2% and 61.3±5.19% for control ($p<0.001$). However, of explants that produced embryos, the number of regenerated embryos per explant was significantly higher ($P<0.001$) for p50AC media at 9.71±0.602 embryos/explant on average, compared to 6.29±0.427 embryos/explant. The increase to somatic embryos/explant is also reflected in the increased growth of PEMs on P50AC cultures, with 62.8±6.41% of P50AC explants showing at least one PEM and 32.5±6.57% for p10 control. Of those explants showing PEMs, multiple PEMs were occasionally observed. Of the explants that had regenerated at least one somatic embryo, the number of PEMs per explant was significantly higher on p50AC media at 1.25±0.120 PEM/explant, compared to 0.719±0.0689 for control.

Callus tissue was regenerated in both experimental and control cultures, however callus was far more prevalent in P50AC media with 34.2±5.40% of cultures showing some level of callogenesis, compared to 7.97±3.87% for p10 control. In addition, some organogenic root growth was observed, mostly in p50AC media with 7.00±1.95% of explants showing root growth and far fewer in p10 at 0.230±0.230% of explants showing organogenic root growth. Several mature embryos were observed exclusively in p50AC media. Similar callus growth was observed in callus cultures. The concentration of exogenous NAA was shown to affect the type of tissue that was regenerated, with low concentrations promoting root growth and higher concentrations promoting callus growth (Gueye et al., 2009). Small preliminary experiments with açaí cultures found that little tissue growth, except for some callogenesis, was induced when AC was added without increasing auxin concentration, whereas increasing auxin concentration without adding AC led to rapid tissue oxidation (data not shown). *Allium cepa* cultures produced roots when cultured with charcoal medium where no roots had been produced before (Fridborg and Eriksson, 1975). In the same report, *Glycine max* and *Haplopappus gracillis* culture growth was inhibited by charcoal, leading the authors to suggest that AC removes substances such as auxin from the media. Indeed, Weatherhead et al., (1978) showed that AC could absorb NAA up to 300mg/L, in addition to other substances, including cytokinins. Canary Island date palm cultures grew increased amounts of callus and had less embryogenic potential when cultured with activated charcoal (Huong et al., 1999). Loss in embryogenic potential might be linked with the

decreased effectiveness of auxin in the presence of activated charcoal. Likewise, bottle palm (*Hyophorbe leganicaulis*) cultures had higher rates of callogenesis when cultured with AC (Sarasan et al., 2002). Several mature somatic embryos were also found in P50AC media. A common step in somatic embryo differentiation and maturation is a reduction in available auxin, such as in rattan palms (Goh et al., 1999). Taken together, addition of AC and increased levels of picloram provided an environment that provided both the positive effects of AC with the increased level of auxin needed to replace that absorbed by the AC. Future experiments may focus on increasing the picloram concentration slightly higher or lower to observe relative differences in calli and SE production. Optimization would involve finding the auxin concentration high enough to avoid callogenesis, but not enough to cause tissue oxidation.

Table 4-3: Types of tissues resulting from açaí multiplication on MS media with 10µM picloram or MS media with 2.5g/L activated charcoal and 50µM picloram

Media	Explant oxidation (%)	Explants with embryos (%)	Average embryos per explant (#)	Explants with PEMs (%)	Average number PEMs per explant (#)	Explants with callus (%)	Explants with organogenic growth (%)
Control p10	27.9 ±5.2 4%	61.3±5. 19%	6.29±0. 427	32.5±6. 57%	0.719±0. 0689	7.97±3. 87%	0.230±0.2 30%
P50+AC	6.99 ±3.8 2%	88.6±4. 2%	9.71±0. 602	62.8±6. 41%	1.25±0.1 20	34.2±5. 40%	7.00±1.95 %

G3 carbon source

There was not a significant difference between number of somatic embryos regenerated between 'G2' peach palm cultures placed on media with 3% sucrose or the combination of carbon sources (p=0.9909). Other studies which had tested different carbon sources usually found that 3% sucrose was the optimal carbon source (Te-Chato and Hilae, 2007). However, the comparable results suggest that this carbon source mixture is an alternative, but 3% is much simpler to use than a mixture. An average of 473.8 embryos per replicate were regenerated on media with 3% sucrose compared to 482.2 embryos/replicate for the mixture.

G3 salt mixture

No significant difference was detected between the mean number of somatic embryos regenerated on either MS media, 188±44 embryos/replicate, or modified MS media, 183±43.3 embryos/replicate (p=0.904). Similarly, no significant difference was found between juçara culture response on Y3 or MS media (Guerra and Handro, 1998). While certain basal medias have been shown to be preferential, such as MS over WPM media (Mazri, 2013), 'G3' peach palm cultures showed no significant difference.

Polyamine addition

Addition of either 1mM spermine or 1mM spermidine had a significantly negative effect on somatic embryo growth on peach palm cultures (p=0.0264), however there was not a significant difference between either spermine or spermidine treatments. Cultures regenerated on control multiplicationmedia produced 185.8±12.7 embryos per repitions, compared to 145.4±6.38 and 154.4±8.21 embryos per repitions for 1mM spermine and 1mM spermidine, respectively. Unlike the previous reports of increased SE in oil palm with addition of spermidine (Rajesh et al., 2003), neither 1µM spermine or spermidine had a beneficial effect on 'G3' peach palm cultures. In date palm, 100 mg/L exogenous putrescine increased somatic embryo multiplication over media without putrescine (Hegazy and Aboshama, 2010). However, future experiments might investigate the use of putrescine at various concentrations rather than either spermine or spermidine.

Partial desiccation

There was no overall significant difference in number of regenerated somatic embryos among high (p=0.6775) and low (p=0.2535) 'Jirau 3' cultures (Table 4-7). However, low embryogenic cultures partially desiccated for 12 hours produced 62.37% more embryos than untreated cultures. Optimal desiccation times for date suspension cultures showed that 24 hours desiccation les to the highest number of regenerated embryos, however, later times killed the cultures. There was no trend of increasing or decreasing number of somatic embryos regenerated in high embryogenic cultures.

Table 4-4: Number of somatic embryos regenerated by high and low embryogenic 'Jirau 3' peach palm cultures to 0, 6, 12, and 24 hours of partial desiccation.

Number of regenerated somatic embryos by partial desiccation time								
Replicate	High embryogenic cultures				Low embryogenic cultures			
	0	6	12	24	0	6	12	24
1	42	49	44	55	13	15	25	19
2	67	78	68	64	26	32	43	35
3	44	39	42	65	45	56	62	51
4	46	52	43	59	28	34	44	38
5	87	89	72	92	32	37	57	45
Average	57.2	61.4	53.8	67	28.8	34.8	46.2	37.6

Butiá friable callus as a model of a recalcitrant phenotype

No somatic embryos were ever regenerated in any of the tested media or treatments (Table 4-8). The best treatment for maintaining healthy yellow friable butiá callus was either with solid or liquid 10µM picloram MS media. Media with 2.5g/L AC and 50µM picloram supported growth, but to a lesser extent. Media containing 14.7µM 2-ip and 5µM NAA, often used for somatic embryo differentiation and maturation, did not show embryo growth, rather the cultures grew little and turned white and watery. See figures 4-2 A-C for visual descriptions of callus health. Reducing auxin concentration promoted embryo differentiation in coconut (Verdeil et al., 1994) and areca palm (Karun et al., 2004), but not in butiá. While cytokinins have been frequently used to help promote somatic embryo differentiation after induction, such as in date (Abohatem et al., 2011) or oil palm (Taha et al., 2001), this simple modification did not promote SE in butiá cultures Addition of 5µM ABA did not promote SE, rather the cultures showed light oxidation. Substituting 1µM GA3 for the 5µM ABA also did not promote SE. Lack of growth regulators with or without 2.5g/LAC in both the light and dark showed low growth of white waterlogged callus. Half MS macrosalts did not promote SE. Dehydration treatments for 12, 24, and 48 hours did not promote SE, however cultures resumed growth rapidly once placed on multiplication media with 10µM picloram. In an experiment to test the effect of additional GA3 under higher osmotic conditions, it was found that neither 8% sucrose, nor 1µM GA3 induced SE in butiá cultures. The highest rate of growth of yellow callus was with the control, MS media with 10µM

picloram and 3% sucrose, however addition of 1µM GA3 had little effect except that the cultures tended to visually appear less yellow. Butiá cultures placed on MS media with 8% sucrose and 10µM picloram with or without 1µM GA3 had less growth and cultures tended to turn white with some signs of oxidation. However, fine chopping caused cultures to completely die within a week, regardless of growth regulators and in stark contrast to the improved SE in date palm (Othmani et al., 2009). Rather than have any immediate agricultural use, this butiá culture line is a potential model for studying recalcitrance. Future optimization studies might investigate the effect of conditioned media, such as that used for peach palm, on potentially inducing SE. It was found that certain arabinogalactan proteins were found on regions of peach palm somatic embryos that eventually gave rise to new somatic embryos through secondary somatic embryogenesis (Steinmacher et al., 2012). These growth-inducing substances might remain in the media and affect butiá callus.

Table 4-5: Response of recalcitrant butiá callus to various media mixtures and treatments

Media/treatment	Embryos growth	Callus Growth	Callus color
Solid media			
10µM picloram	None	High growth	pale to deep yellow
2.5g/L AC, 50µM picloram	None	Medium growth	pale yellow
14.7µM 2ip, 5µM NAA	None	Low growth	White
14.7µM 2ip, 5µM NAA, 5µM ABA	None	Low growth	White, some oxidation
14.7µM 2ip, 5µM NAA, 1µM GA	None	Low growth	White
No GR, light	None	Low growth	White
No GR, dark	None	Low growth	White
No GR, 2.5g/L AC, light	None	Low growth	White
No GR, 2.5g/L AC, dark	None	Low growth	White
1/2MS media, dark	None	Low growth	White
12 hour partial dehydration	None	Medium growth	Pale yellow
24 hour partial dehydration	None	Medium growth	Pale yellow
48 hour partial dehydration	None	Medium growth	Pale yellow
10µM picloram, 3% sucrose	None	High growth	Pale to deep yellow
10µM picloram, 3% sucrose, 1µM GA	None	High growth	Pale yellow
10µM picloram, 8% sucrose	None	Medium growth	White
10µM picloram, 8% sucrose, 1µM GA	None	Medium growth	White to brown
Fine chopping, 10µM picloram	None	None	Complete Oxidation
Liquid media			
10µM picloram	None	High growth	Pale to deep yellow
No GR	None	Low growth	White
1/2MS media	None	Low growth	White

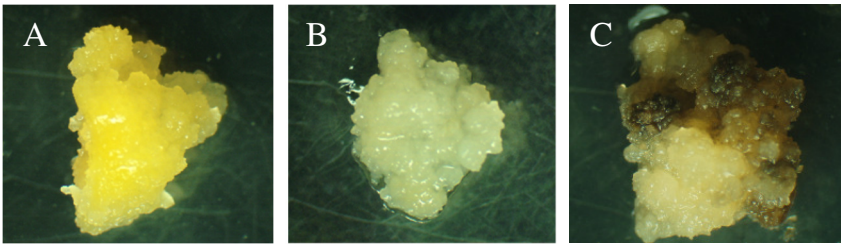

Figure 4-2: Response of butiá callus to (A) MS media containing 10µM picloram; (B) MS media containing 14.7µM 2-ip and 5µM NAA; (C) MS media containing 8% sucrose and 1µM GA3 test

Butiá zygotic embryo SE induction

Butiá zygotic embryo response depended on presence of picloram (Table 4-8). Media without growth regulators promoted germination in 27.5% of embryos, however 67.5% of zygotic embryos had no response and 5% of zygotic embryos were oxidized. Both 300 and 450µM picloram promoted growth of both embryogenic and non-embryogenic callus with 40% and 27.5% response rates, respectively (Figure 4-3 A-D). Just like media without picloram, the majority of zygotic embryos did not respond with a respective 52.5% and 55% lack of response for 300µM and 450µM. While only 7.5% of embryos placed on media with 300µM picloram oxidized, zygotic embryos placed on 450µM picloram had much higher oxidation rates at 17.5%. Structures similar to those found in embryogenic peach palm tissue were observed, by no distinct somatic embryos have yet to be developed.

Table 4-6: Response of Butia eriospatha zygotic embryos to MS media containing 0, 300 or 450µM picloram

Media	Culture growth	Germination	Oxidation	No response
No PGR	0%	27.50%	5%	67.50%
P300	40%	0%	7.50%	52.50%
P450	27.50%	0%	17.50%	55%

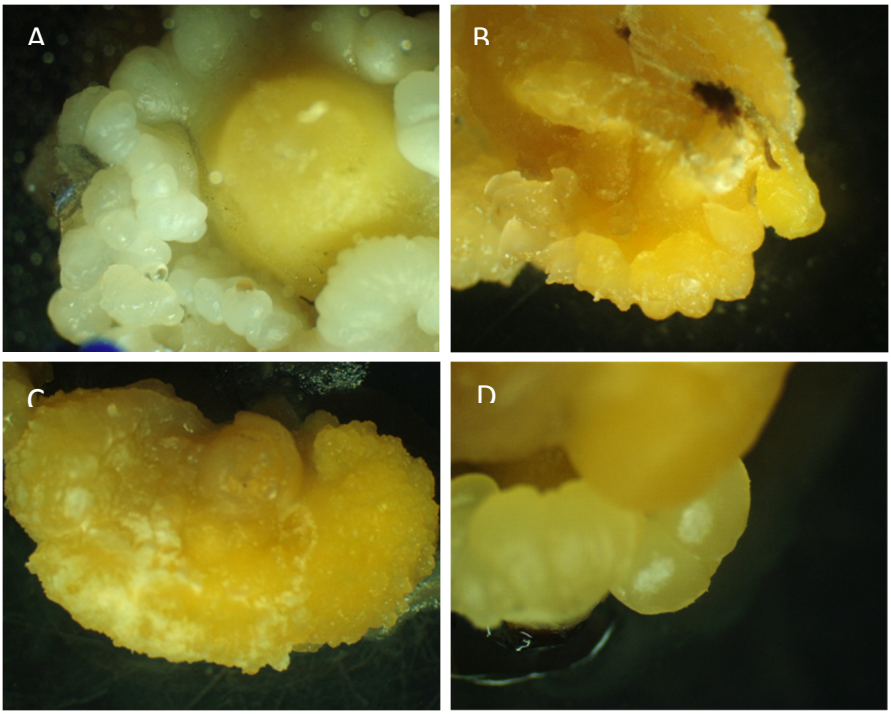

Figure 4-3: Tissue growth from zygotic embryos placed on MS media with 300µM picloram; (A) Nodular structures growing in an organized outward curl from the callus center, similar in morphology to peach palm low embryogenic potential tissue; (B) Unorganized yellow nodular tissue; (C) hard compact callus; (D) Smooth lobed structures found near organized curling lobbed structures mentioned in figure 4-3A. Both structures are highly associated with embryogenic peach palm structures.

Conclusions

Improved Açaí *in vitro* culture multiplication was achieved using MS multiplication media with 2.5g/L AC and 50µM picloram as shown through reduced culture oxidation and increased amounts of somatic embryo and PEM regeneration. However, this came at the expense of increased callus growth, as well as organogenic root growth. The new salt mixture was shown to be as effective as MS media in 'G3' line peach

palm somatic embryo multiplication. Similarly, mixtures of carbon sources were found to be as effective as 3% sucrose on ‘G2’ peach palm peach palm somatic embryo multiplication. Both 1µM spermidine and spermine were found to have a slight negative effect on ‘G3’ peach palm peach palm somatic embryo multiplication. Partial dehydration was not found to affect ‘Jirau 3’ peach palm line multiplication. No somatic embryos were regenerated from friable butiá callus cultures, although SE was induced using zygotic embryos.

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5. Concluding remarks

This work attempted to expand the understanding of somatic embryogenesis through literature review of somatic embryogenesis in palms to identify areas of interest, protoplast culture to develop a means of regenerating single individuals without damaging plants and open the possibility of creating hybrid palms, biochemical analysis of tissue with different levels of embryogenic competence to understand the relation between nutrient amounts and potential for growth, and media optimization in order to build trends with results from the literature to provide more reliable means of somatic embryo induction and multiplication. Protoplasts are a complex system with many avenues for future research, however success is often measured in if plants were regenerated. In this regard, there was no success. However, it is important to remark that protoplast cultures, like somatic embryo induction, takes weeks to months and under proper conditions. The factors tested here show several key results: the use of short amounts of protoplast isolation incubation on an orbital shaking can be used to generate large numbers of viable protoplasts, the requirement of auxin for stimulating plant cells to reenter the cell cycle earlier, and that either agarose or alginate embedding can be used to generate colonies of regenerating palm cells. Future work must expand on this toward generating plants, such as through culturing protoplasts on an orbital shaker, increased cell culturing density, and use of nurse cultures, all of which have resulted in fertile plant regeneration in non-palm species. Biochemical analysis showed little difference between high-embryogenic cultures and non-embryogenic cultures while showing a large difference between these two types of tissue and low embryogenic cultures. These data, along with morphological observations, point to possible organogenesis, however this would require further investigating to confirm. To make better sense of this phenomenon, further studies of biochemical difference between tissues should select specific structures, such as the lobed structures in low embryogenic cultures, globular embryos alone, or specific sections of non-embryogenic culture fibrous masses. Optimization of all three species had both a large success, the açaí multiplication improvement, and a major lack of success, culture of butiá friable callus. In the course of the over twenty types of media and treatments attempted, none led to somatic embryo growth. Additional means of optimization could be attempted in

the future, but this type of tissue would be best suited for genetic analysis of recalcitrance. Of almost all the methods used in palm literature to differentiate somatic embryos, the vast majority were attempted, without generating a single embryo. This tissue line might serve as a highly recalcitrant model for studying the roots of culture recalcitrance. Açaí culture, however, not only decreased tissue oxidation but increased the number of regenerated somatic embryos. This work not only improved culture multiplication, but also supported previous observations in which the effect of auxin was diminished in media with activated charcoal and this effect can lead to callogenesis and organogenesis. Future experiments with açaí should focus on maturation and conversion into plants.